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The Incidence of Vibrio Species in Louisiana and Maryland Oysters (Crassostrea Virginica).

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The incidence of *Vibrio* species in Louisiana and Maryland oysters (*Crassostrea virginica*)

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THE INCIDENCE OF *VIBRIO* SPECIES IN LOUISIANA AND MARYLAND OYSTERS (*CRASSOSTREA VIRGINICA*)

A dissertation

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in

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by
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DEDICATION

To my parents, who were determined that each of their children would receive a higher education and instilled that goal in us early.

ACKNOWLEDGMENT

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ABSTRACT

Vibrio species, normal inhabitants of the sea and of shellfish harvested from marine or brackish waters, have been established as the causative agents of serious human infections. An investigation to isolate and identity Vibrio species in oysters collected from eight Louisiana commercial oyster beds was conducted. A selection of 127 vibrio isolates and 46 reference strains, including all known Vibrio species, were subjected to a bacteriological identification scheme of 46 parameters. When the data from the wild strains and the reference strains were analyzed by the numerical taxonomy program, TAXAN Version 3.0, six major phena were evident. Four of the phena contained Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio cholerae, Vibrio mimicus, Vibrio furnissii, and Vibrio Group 521. Genetic relatedness studies showed one of the other phena to be composed of isolates of Vibrio damsela. The remaining major phenon appears to be a previously undescribed Vibrio group.

Because of the severity and rapidity of the disease process in Vibrio vulnificus infections and because of the high mortality rates associated with these infections, a rapid serological method for V. vulnificus identification was developed. Species-specific anti-V. vulnificus flagellar (H) monoclonal antibody coagglutination reagents were sent to laboratories throughout the United States and Canada to be assessed for specificity. One of the user laboratories reported that fifteen Vibrio strains, isolated

from Eastern seaboard oysters, agglutinated in these reagents but did not conform to established V. vulnificus bacteriological profiles. These findings suggested that the reagents may not be species-specific. DNA from representative oyster isolates which produced agglutination reactions were examined for thermal denaturation midpoint temperature, mol% G + C content, and DNA-DNA reassociation with DNA from V. vulnificus biogroups 1 and 2. High percentages of relative reassociation with V. vulnificus reference strains indicate that the H coagglutination reagents exhibit a high degree of specificity for V. vulnificus isolates, even those showing aberrant phenotypic characteristics.

INTRODUCTION

Members of the genus Vibrio comprise a large, somewhat diverse group of organisms which are mostly halophilic in nature and are abundant in the environment, especially in shellfish and marine waters. This genus is one of four genera in the family Vibrionaceae. The other three genera are Aeromonas, Photobacterium, and Pleisomonas. In 1972, the Subcommittee on Taxonomy of Vibrios of the International Committee on Systematic Bacteriology accepted the following description for the genus Vibrio:

> "Gram-negative, asporogenous rods which have a single, rigid curve or which are straight. Motile by means of a single, polar flagellum. Produce indophenol oxidase and catalase. Ferment glucose without gas production. Acidity is produced from glucose by the Embden-Meyerhof glycolytic pathway. The guanine plus cytosine in the deoxyribonucleic acid of Vibrio species is within the range of 40 to 50 moles percent." (Anonymous, 1972).

Today, nearly twenty years later, this definition has been expanded and changed, almost completely, as a result of extensive investigations of these intriguing organisms. It is now known that vibrios are motile by means of monotrichous or multitrichous polar flagella in liquid media and may synthesize shorter lateral flagella when grown on solid media. They are facultative anaerobes capable of both fermentative and oxidative metabolism. Of the presently

recognized Vibrio species, Vibrio metschnikovii and Vibrio gazogenes are negative for production of indophenol oxidase and Vibrio furnissii and V. gazogenes produce gas from glucose fermentation. The base composition of deoxyribonucleic acid (DNA) of members of the genus Vibrio, as measured in moles percent guanine-plus-cytosine (mol% G + C), is 38-51 (Baumann et al., 1984).

The genus Vibrio was once a large group of poorly characterized organisms, but, recently, the group has become much more well defined. "Nonfermentative vibrios," "anaerobic vibrios," and "microaerophilic vibrios" have been moved to other genera such as Campylobacter (Vibrio fetus), Wolinella (Vibrio succinogenes), Pseudomonas, and Alteromonas (Baumann et al., 1981; Farmer et al., 1985). Presently, there are 34 species in the genus Vibrio and several unnamed Vibrio groups (Farmer, 1989, personal communication). The Vibrio species were originally defined according to their bacteriological characteristics and this approach to identification is still widely used. Recent investigations in which molecular assays were employed such as DNA-DNA hybridization, amino acid sequencing, cellular fatty acid profiles, 5S ribosomal ribonucleic acid (rRNA) sequencing and numerical taxonomic studies generally support the delineation between the presently recognized 34 Vibrio species. This support is not complete however. Based on 5S rRNA analyses, MacDonell and Colwell (1985) and Pillidge et al. (1987) believe that Vibrio anguillarum, Vibrio damsela,

Vibrio pelgaius, and Vibrio aestuarianus should be placed in new and separate genus, Listonella. They further indicate that Vibrio fischeri and Vibrio logei should be transferred to the genus Photobacterium. Because these changes have not been widely accepted among investigators, the Vibrio species in question will be considered members of the genus Vibrio in the investigation reported herein.

Of the 34 currently recognized Vibrio species, 11 have been implicated in human infections. Vibrio cholerae is the best known of these and is the etiological agent of the pandemics of cholera in countries worldwide. The Vibrio cholerae serological varieties are grouped by virtue of cell wall (O) antigen differences and by the ability to produce cholera toxin. Toxigenic V. cholerae group 01 is the causative agent of epidemic cholera but does not cause extraintestinal infections. Nontoxigenic V. cholerae 01 and strains of non-01 V. cholerae have been associated with both gastrointestinal and extraintestinal infections, such as wound infections, ear infections, septicemia, and cholecystitis (Blake, 1983; Blake et al., 1979; Johnston et al., 1983; Morris et al., 1981). Vibrio fluvialis, V. furnissii, V. hollisae, V. mimicus, and V. parahaemolyticus have been implicated primarily in cases of gastroenteritis, but they have also been encountered in extraintestinal infections (Farmer et al., 1985; Morris et al., 1982; Shandera et al., 1983; Tacket et al., 1982). Vibrio alginolyticus, V. damsela, V. metschnikovii, and V. vulnificus have been recovered from extraintestinal

infection sites which include wounds, ear, eye, or lung exudate following trauma and exposure to seawater and/or shellfish (Blake et al., 1980; Hollis et al., 1976; Jean-Jacques et al., 1981; Morris et al. 1982). The single case of infection due to Vibrio cincinnatiensis reported to date was manifested as bacterial sepsis and meningitis in an immunocompetent adult without exposure to seafood or seawater (Bode et al., 1986).

Vibrio vulnificus, an organism of unusual virulence is capable of causing soft tissue infections and fatal septicemias often accompanied by skin lesions on the extremities or trunk as a direct result of sepsis (Tacket et al., 1984). V. vulnificus produces an extremely potent vascular permeability factor and experimental evidence suggests the organism can rapidly penetrate subcutaneous tissue and gut mucosa (Poole et al., 1978). Both clinical and environmental strains of V. vulnificus produce a toxin capable of lysing human erthrocytes (Johnson et al., 1981). A significant correlation between consumption of raw oysters and fatal V. vulnificus septicemia and an association between contact with seawater or shellfish and V. vulnificus wound infections has been shown by Blake et al. (1979, 1980).

Although Vibrio species have been isolated in various geographic localities in the United States, the frequency of Vibrio infections is greater in the coastal states of the Atlantic seaboard and the Gulf coast (Janda et al., 1988). Persons infected orally by Vibrio species, almost without

exception, have ingested raw or undercooked seafood or seafood that was recontaminated after being cooked. Seafoods that have been implicated in foodborne Vibrio infections are raw oysters and fish and cooked crayfish, crabs, shrimp, lobster, and clams (Blake, 1983).

The seafood industry is virtually unregulated at this time. There are regulations in place, predicated on coliform levels and aerobic bacterial counts, for commercial molluscan shellfish, but there are no regulations which govern shrimp, crabs, crayfish, squid, or fin fish in commerce. In fact, at certain times of the year, a citizen may buy shrimp or crayfish off the back of a pickup truck along almost any major thoroughfare in south Louisiana. The regulations on oysters and clams appear to be inadequate. Several major lawsuits have been filed as a result of illnesses and deaths resulting from V. vulnificus infections traceable to ingestion of raw oysters. As a consequence, the seafood industry will soon be subject to federal regulations. Whether these regulations will come under the auspices of the Food and Drug Administration (FDA) or the United States Department of Agriculture (USDA) is a point of controversy at this time between these two regulatory agencies.

Because raw oysters are often consumed, the industry is investigating depuration techniques to remove the microbial flora from the oyster. Guidelines for depuration have been set by the Interstate Shellfish Sanitation Conference. Briefly, the depuration process involves thorough washing of

the oysters before placing them in a tank of constantly recirculating water. The recirculated water is "sterilized" by ultraviolet light or ozone. The oysters must remain in the tank for a minimum of 48 hours and must be fed, while in the tank, because the depuration event is dependent upon each oyster "pumping" water as it filter feeds. Oyster depuration presents two problems: 1) Ozone irritates the oyster and causes it to cease feeding and 2) V. vulnificus can be recovered from tissue parts of depurated oysters, especially the mantle (Nell Roberts, 1990, personal communication). Though depuration techniques have not been refined at this point in time, the National Oceanic and Atmospheric Administration has awarded a grant to investigators at the University of Florida and at Nicholls State University of Louisiana to work with commercial seafood plants in developing standard methods for oyster depuration.

Presently, we are on the brink of regulation of a major enterprise, the seafood industry. Because vibrios have been specifically and heavily implicated in illness due to consumption of raw seafood, it is imperative that rapid identification techniques be developed and that information on Vibrio species and their association with seafood be provided to public health officials, regulatory agencies, and seafood industry personnel. It is hoped that the information gained from the investigations presented in this dissertation will be useful in the effort to provide the public with safe, enjoyable seafood products.

CHAPTER I Identification of *Vibrio* species in Louisiana oysters--discovery of a new *Vibrio* group.

INTRODUCTION

Louisiana leads its sister Gulf states in oyster production by producing an average of 9,000,000 pounds of oyster meat annually, and usually ranks second nationally only to Maryland. The lowest Louisiana catch on record is a total of 1,189,000 pounds in 1880 while the highest catch, a total of 13,700,000 pounds was recorded in 1984. The oyster industry relies almost entirely on the American oyster, Crassostrea virginica. The annual dockside value of the harvested oysters is from $6,000,000 to $10,000,000 (Anonymous, 1987).

The oyster harvesting waters of Louisiana are divided into two regions, those areas which are leased to private citizens and those areas which are state-controlled. Privately leased areas comprise approximately 264,000 acres and are dispersed in estuaries from the Mississippi-Louisiana boundary line on the east to Vermilion Bay on the west. The state has approximately 690,000 acres under its jurisdiction. Of this, 16,453 acres are maintained as "Seed Ground Reservations." Commercial fishermen may enter the oyster seed grounds at designated times of the year to retrieve seed oysters (1 to 3 inches) for relocating to better growing areas. On the western state boundary, an area of 6,737 acres in Calcasieu Lake comprises another state-controlled oyster growing area. Of the remaining 650,000 plus acres under state management, only approximately 200,000 acres are dredged for oysters

(Anonymous, 1987). Unfortunately, an estimated two-thirds of Louisiana oysters are growing in waters closed to harvest because of microbial pollution.

Ingestion of raw or undercooked shellfish is known to result in enteric diseases which may be either bacterial or viral in nature (Dupont, 1986; Morse et al., 1986; Janda et al., 1988). Examples of such diseases are typhoid fever, hepatitis A infection, Norwalk virus infection, cholera, and gastroenteritis caused by Campylobacter species and Vibrio species other than Vibrio cholerae. Traditionally, it has been assumed that these diseases have been transmitted through the fecal-oral route with shellfish acting as vectors of the disease agents. For this reason, fecal coliforms are considered standard sanitary indicator organisms. Regulatory authorities rely on fecal coliform levels, as measured by "most probable number" (MPN) assays, and aerobic plate counts (APC), which indicate total heterotrophic bacterial numbers, as indices of sanitary quality in seafood and shellfish harvesting waters.

Guidelines for regulation of shellfish harvesting areas are provided by the National Shellfish Sanitation Program Manual of Operations, Part I (Anonymous, 1988). According to these guidelines, an oyster harvesting area will fall into one of five possible classifications:

1. Approved Area. This area has a geometric mean fecal coliform MPN of $\leq$14 per 100 milliliters (ml) of water and not more than 10% of the water samples exceed an MPN of 43/100 ml in 5-tube MPN tests.

2. Conditionally Approved Area. This area has the same numerical guidelines as the Approved Area but may be closed at times as the result of on-site sanitary surveys by health officials.
3. Restricted Area. This area has a geometric mean fecal coliform MPN of $\leq$88/100 ml and not more than 10% of the water samples exceed MPNs of 260/100 ml. The oysters from a restricted area must be subjected to depuration and approval by the health department.
4. Conditionally Restricted Area. This area is similar to the restricted area but may be closed due to a predictable pollution event such as flooding.
5. Prohibited Area. No oysters may be harvested from a prohibited area. There are no numerical guidelines for this area, but an obvious source of pollution exits such as a nearby sewer outlet.

Most oyster harvesting areas in Louisiana are subject to management by flood stage. For example, all oyster beds in Cameron Parish are closed when the flood stage of the Calcasieu River, as measured at Kinder, Louisiana, reaches a depth of 12 feet and for two weeks after the water level falls below 12 feet.

Guidelines for regulation of fresh and frozen oysters at the wholesale market level are stated in the National Shellfish Sanitation Program Manual of Operations, Part II (Anonymous, 1988). After being identified as having been

produced under the general sanitary controls of the National Shellfish Sanitation Program, the oysters are placed into one of two categories:

1. Satisfactory. Fecal coliform density of not more than 230 MPN per 100 gram and 35 °C plate count of not more than 500,000 colony-forming units (CFU) per gram will be acceptable without question.
2. Conditional. Fecal coliform density of more than 230 MPN per 100 grams and/or 35 °C plate count of more than 500,000 CFU per gram will constitute a conditional sample and may be subject to rejection by the state shellfish regulatory authority.

In Louisiana, regulations on oysters and oyster harvesting waters are administered jointly by the Department of Health and Hospitals and the Department of Wildlife and Fisheries. Personnel of the Department of Health and Hospitals conduct bacteriological testing of oyster meat and oyster growing waters and conduct on-site sanitary surveys. Department of Wildlife and Fisheries personnel carry out enforcement procedures.

While pathogenic organisms indeed can be transmitted by fecal contamination of shellfish and shellfish growing waters, it has been shown that fecal coliform levels and heterotrophic bacteria levels do not accurately predict shellfish contamination by viruses or vibrios (Portney et al., 1975; Gerbia et al., 1979; Roberts et al., 1982; Hood et al., 1983; Roderick et al., 1984). A large study was conducted by the Louisiana Department of Health and

Hospitals in conjunction with the Department of Microbiology at Louisiana State University in which 963 market level shellstock and shucked oyster, crab, and crayfish samples were assayed for fecal coliform levels, aerobic bacteria levels, and presence or absence of five pathogenic Vibrio species. The Vibrio species under test in that study were V. cholerae, V. fluvialis, V. mimicus, V. parahaemolyticus, and V. vulnificus. The results of the study indicated that, in the three types of seafood products being assayed, no correlation exists between the presence of those five pathogenic Vibrio species and total bacterial numbers or levels of standard sanitary indicator organisms (Ford et al., 1984 unpublished data).

Other than diseases which are gastrointestinal in nature, several Vibrio species are agents of extraintestinal infections. The most well-known of these is V. vulnificus which, in the realm of extraintestinal disease, is most noted for severe infections of preexisting wounds following exposure to seawater or of puncture wounds incurred while handling raw shellfish (Hollis et al., 1976; Blake et al., 1979; Kelly et al., 1980; Beckman et al., 1981; Tacket et al., 1984).

Certain Vibrio species are pathogens of fish and shellfish. Vibrio tubiashii is one such notable pathogen of bivalve mollusks. This organism manifests itself as the causative agent of fatal epizooties among larval and juvenile stocks in bivalve hatcheries (Tubiash et al. 1965, 1970).

Roberts et al. (1982) and Barbay et al. (1984) reported that Vibrio species are indigenous in the Louisiana coastal environment. The major focus of those investigations was to isolate and identify the human pathogens V. cholerae, V. parahaemolyticus, V. fluvialis, and V. vulnificus. One might surmise then that any or all Vibrio species could be native microflora in Louisiana coastal waters.

The present investigation, to ascertain exactly which Vibrio species are present in Louisiana commercial oysters, was undertaken as a result of four important facts. First, the multimillion dollar oyster industry is important to the economy of the State of Louisiana. Second, the presence of certain pathogenic Vibrio species has been confirmed in Louisiana oysters. Third, vibrio levels fluctuate independently of fecal coliform levels and heterotrophic bacterial counts which are still used as sanitary indices for seafood and shellfish harvesting waters. Lastly, the consumption of "oysters-on-the-halfshell" is still very much in vogue.

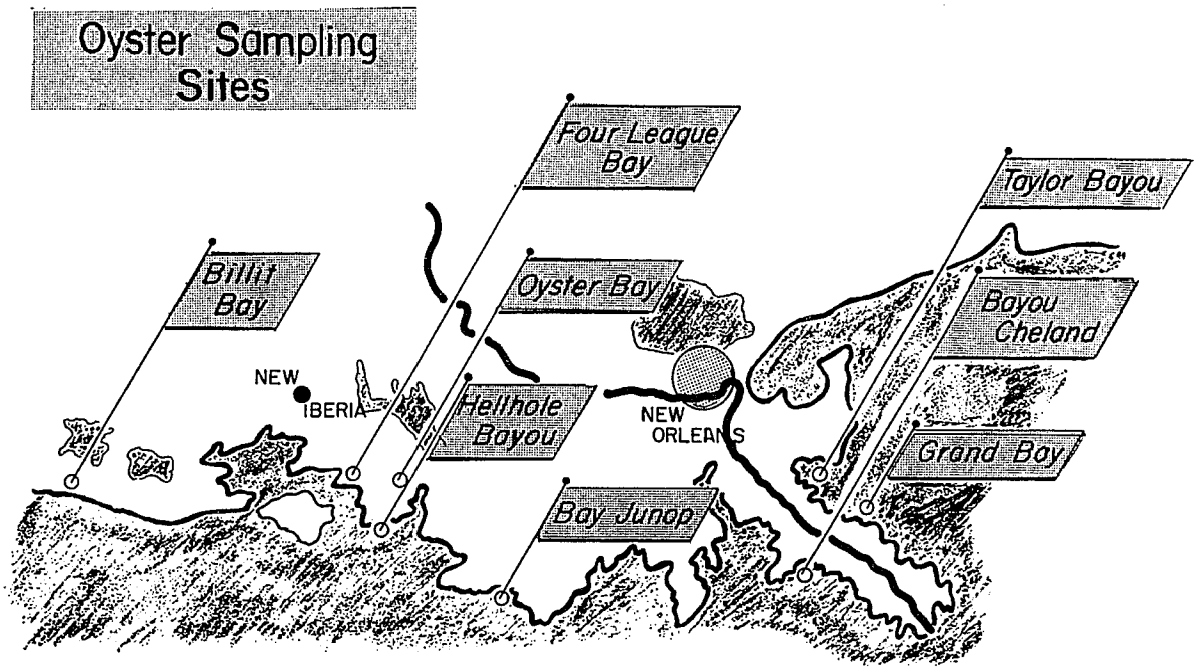
MATERIALS AND METHODS

Shellfish harvest sites. Oysters used in this study were harvested from March, 1985 through July, 1986, from eight commercial fishing areas in south Louisiana. The oysters were harvested from beds located in Bay Junop, Bayou Cheland, Billet Bay, Four-League Bay, Grand Bay Dularge, Hellhole Bayou, Oyster Bayou, and Taylors Bayou (Fig. 1). Water temperature ranged from a low of 13 ^{o}C in the winter months to a high of 29 ^{o}C in the summer months. Water salinity ranged from 7 parts per thousand to 27 parts per thousand.

Oysters were harvested by personnel from the Gulf Coast Research Laboratory, Oceans Springs, Mississippi. The technicians traveled on boats with commercial fishermen, harvested the oysters, and packed them into coolers at $<$10 ^{o}C for transport to the laboratory. Oysters harvested from Louisiana waters did not reach the laboratory in Mississippi until midnight the day they were harvested; therefore, bacteriological testing was not initiated until the following day.

Isolation of vibrios. Samples consisted of 10-12 oysters each, approximately 200 grams of oyster meat per sample. Each sample was weighed in a sterile jar and blended with an equal volume of phosphate buffered water (KH_2PO_4 34 g, distilled water 1000 ml, pH 7.2). Each sample was enriched in alkaline peptone broth for 24 h at 35 ^{o}C.

Figure 1. Oyster sampling sites at eight commercial fishing areas in south Louisiana.


Oyster Sampling Sites
Four League Bay
Taylor Bayou
Billit Bay
Oyster Bay
Bayou Cheland
NEW IBERIA
Hellhole Bayou
NEW ORLEANS
Grand Bay
Bay Junop

The alkaline peptone broth formulation was (g/l): peptone, 10; NaCl, 10; pH 8.5. Each 24 h enrichment was streaked to thiosulfate-citrate-bile salts-sucrose agar (TCBS). Sucrose positive and negative colonies were picked to T_1N_1 agar slants. This medium consisted of (g/l): tryptone, 10; NaCl, 10; agar, 15. A random selection of 127 isolates, in groups of 50 each, were sent to the Department of Microbiology, Louisiana State University.

Reference strains. Bacteriological, numerical taxonomic, and genetic analyses were carried out on 46 Vibrio reference strains concurrently with and under the same test conditions as analyses of the wild strains. The reference strains used and the sources from which they were obtained are listed on Table 1.

Phenotypic characterization of wild isolates and reference strains. Each bacteriological assay was conducted at 30 ^{o}C except the test for bioluminescence which was carried out at room temperature and the test to ascertain the ability of isolates to grow at 42 ^{o}C.

Sodium chloride was added to give a final concentration of 1% to each medium prepared from commercial dehydrated stock, except the medium used in the salt tolerance assay. An electrolyte supplement described by Furniss et al. (1978), which consisted of (g/l): NaCl, 100; $MgCl_2 \cdot 6H_2O$, 40; KCl, 40, was added to each prepared medium. The electrolyte supplement was added at a concentration of 0.1 ml supplement per 1 ml medium, which brought the final NaCl concentration to 2%. Most of the media used in this study

Table 1. *Vibrio* reference strains used in the bacteriological, taxonomic, and genetic analyses and the sources from which they were obtained.

Reference strain	Source
Vibrio aestuarianus	ATCC 35048[a]
Vibrio alginolytics	ATCC 33787
Vibrio anguillarum	ATCC 19264
Vibrio anguillarum	ATCC 14181
Vibrio campbellii	ATCC 25920
Vibrio carchariae	ATCC 35084
Vibrio cholerae Ogawa	ATCC 14035
Vibrio cholerae Inaba	LA 5875[b]
Vibrio cholerae serotype *albensis*	NCMB 41[c]
Vibrio cincinnatiensis	ATCC 35912
Vibrio Group 511 (*V. cincinnatiensis* xylose-negative)	CDC[d]
Vibrio costicola	NCMB 701
Vibrio damsela	ATCC 35083
Vibrio diazotrophicus	ATCC 33466
Vibrio Group 510 (*V. diazotrophicus* arginine-negative)	CDC
Vibrio fischeri	NCMB 1281
Vibrio fluvialis	ATCC 33810
Vibrio furnissii	ATCC 35016
Vibrio gazogenes	ATCC 29988
Vibrio harveyi	CMB 1280
Vibrio hollisae	ATCC 33564
Vibrio logei	ATCC 29985
Vibrio marinus	ATCC 15381
Vibrio mediterranei	ATCC 43341
Vibrio metschnikovii	P206
Vibrio mimicus	ATCC 33653
Vibrio natriegens	ATCC 14048
Vibrio nereis	ATCC 25917
Vibrio nigrapulchritudo	ATCC 27043
Vibrio ordalii	ATCC 33509
Vibrio orientalis	ATCC 33934
Vibrio parahaemolyticus	ATCC 10136

Continued--

Table 1--continued

Reference strain	Source
Vibrio pelagius I	ATCC 25916
Vibrio pelagius II	Unknown
Vibrio proteolyticus	MCMB 13126
Vibrio splendidus I	ATCC 33125
Vibrio splendidus II	ATCC 25914
Vibrio tubiashii A	ATCC 19105
Vibrio tubiashii O	ATCC 19106
Vibrio tubiashii J	ATCC 19109
Vibrio vulnificus biogroup 1	ATCC 27562
Vibrio vulnificus biogroup 2	ATCC 33147
Vibrio Group 512	CDC
Vibrio Group 521	CDC
Vibrio Group 522	CDC
Baumann Group E3	ATCC 33523

a, ATCC, American Type Culture Collection, Rockville, Maryland

b, LA, Louisiana isolate

c, NCMB, National Collection of Marine Bacteria, Aberdeen, Scotland

d, CDC, Centers for Disease Control, Atlanta, Georgia

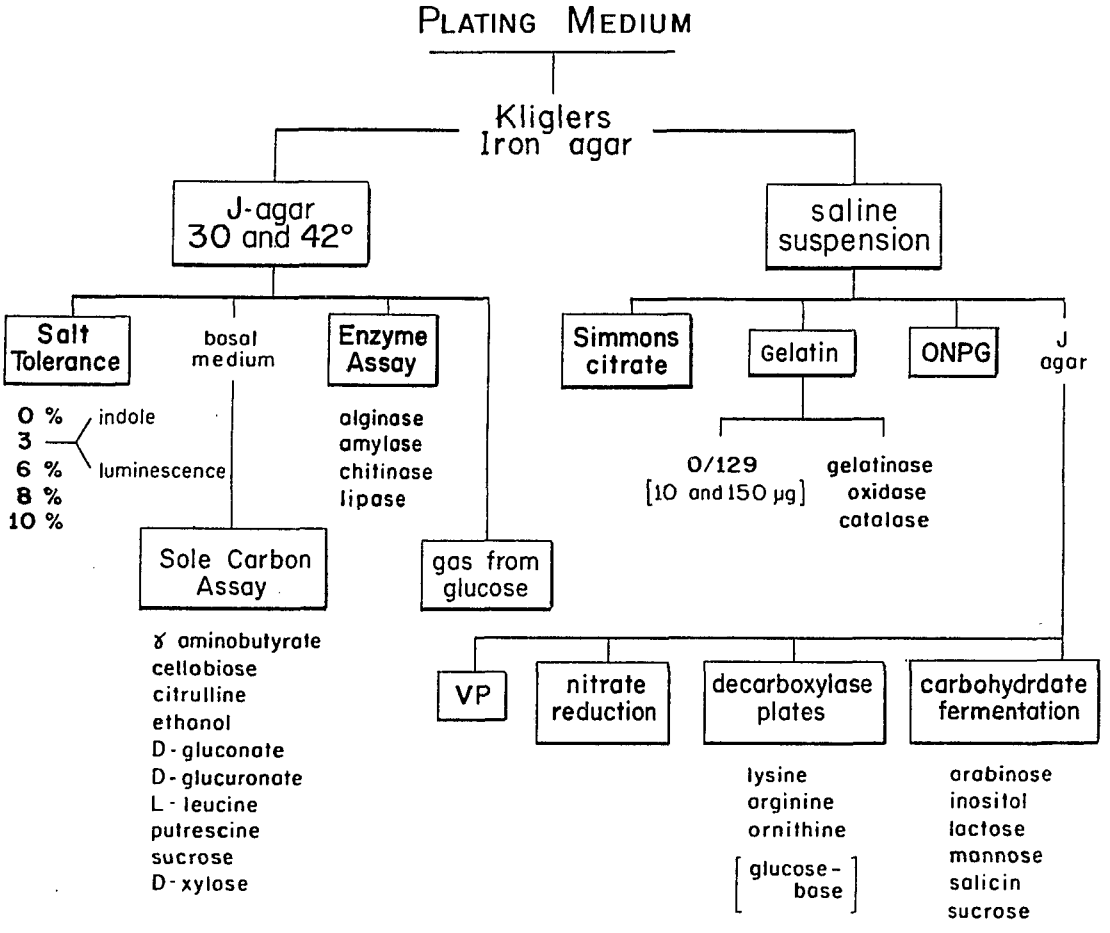
were formulated from individual ingredients and their formulae are listed below.

The bacteriological scheme followed for strain identification is shown on Fig. 2. Upon receipt, each isolate was suspended in 0.15 M NaCl and streaked onto TCBS agar plates (Oxoid U.S.A., Inc., Columbia, Maryland). Following 24 h - 48 h incubation, sucrose positive and negative colonies were picked to Kliglers Iron Agar (KIA) (Difco Laboratories, Detroit, Michigan). After overnight incubation, growth on the KIA slants was scored for sugar fermentation reactions, gas production and production of H_2S. Growth from each KIA slant was then streaked onto two J-Agar slants, stabbed into a Maintenance Medium deep, and saline suspensions were prepared from each isolate. The J-Agar formulation was (g/l): nutrient broth, 25; NaCl, 22.5; KCl, 1; $MgCl_2 \cdot 6H_2O$, 4; agar, 15. The Maintenance Medium formulation was (g/l): tryptose, 8; NaCl, 12.5; nutrient broth, 4; agar, 4; electrolyte supplement, 100 ml; distilled water, 900 ml.

Citrate utilization. The ability of an isolate to utilize citrate was determined by streaking the isolate from the saline suspension onto Simmons Citrate agar (Difco). Change in color of the medium from green to blue, within a 7 day time period, indicated an alkaline reaction and was scored as positive.

Decarboxylase and dihydrolase reactions. To determine the ability of each isolate to decarboxylate L-lysine and L-ornithine and to dihydrolyze L-arginine, the amino acids

Figure 2. Bacteriological scheme for Vibrio species identification.


PLATING MEDIUM
Kliglers Iron agar
J-agar 30 and 42°
saline suspension
Salt Tolerance
bosal medium
Enzyme Assay
Simmons citrate
Gelatin
ONPG
J agar
0 %
3
6 %
8 %
10 %
indole
luminescence
alginase
amylase
chitinase
lipase
O/129 [10 and 150 µg]
gelatinase
oxidase
catalase
Sole Carbon Assay
gas from glucose
ϒ aminobutyrate
cellobiose
citrulline
ethanol
D-gluconate
D-glucuronate
L-leucine
putrescine
sucrose
D-xylose
VP
nitrate reduction
decarboxylase plates
carbohydrdate fermentation
lysine
orginine
ornithine
[glucose-base]
arabinose
inositol
lactose
mannose
salicin
sucrose

(Sigma Chemical Co., St. Louis, Missouri) were added to Moeller's Decarboxylase Medium Base Broth (Difco) to give a 1% concentration. A 2% concentration of agar (Difco) was added to the medium. After sterilization, the medium was pipetted into each well of a 24-well sterile tissue culture cell well plate (Corning Glass Works, Corning, New York). Two milliliters of medium were added to each well and overlayed with 0.5 ml sterile mineral oil. Twenty-four isolates were inoculated onto each plate at one time with an inoculation device to which 24 loops had been attached. The decarboxylase substrate was inoculated by touching the loops to a 24-well plate which contained vibrio isolates growing on the surface of J-Agar, and then stabbing the decarboxylase - dihydrolase media through the oil and media to the well floor. Agar and oil were removed from each of the loops by dipping them into a mixture of sand and 95% ethanol and then flaming. The decarboxylase - dihydrolase cultures were incubated for 4 days. A positive reaction was determined by a change in the indicator (brom cresol purple) in the medium to acid and then back to alkaline. Each isolate was also inoculated into decarboxylase medium without added amino acids.

Enzyme assay. Quad plates (Baxter, McGraw Park, Illinois) were used for alginase, amylase, chitinase and lipase assays. Six milliliters of each of the substrates identified below were added to one of the partitions of each quad plate.

(i) Alginase. Production of alginase was determined

by the method of West and Colwell (1984).

(ii) Amylase. Production of amylase was determined by the method of Cowan (1974) and MacFaddin (1980). Grams iodine was used to detect zones of clearing around growth.

(iii) Catalase. Production of catalase was determined by dropwise addition of 3% hydrogen peroxide (Hunt Products Company, Dallas, Texas) onto growth on a gelatin agar plate (Gelatin medium formula is given below). Formation of gas bubbles indicated catalase activity.

(iv) Chitinase. Production of chitinase was determined by the method of West and Colwell (1984).

(v) Gelatinase. Hydrolysis of gelatin was determined by streaking each isolate onto gelatin agar. The growth following 24 h incubation was flooded with mercury bichloride. The appearance of clear zones around bacterial growth indicated gelatinase production. Gelatin agar was prepared from (g/l): neopeptone, 4; yeast extract, 1; gelatin, 15; NaCl, 30; agar, 15. The gelatin developer was composed of: $HgCl_2$, 3.75 g; conc. HCl, 5 ml; distilled water, 35 ml.

(vi) Lipase. Production of lipase was determined by the method of West and Colwell (1984).

(vii) Oxidase. Production of oxidase was determined by the addition of a 1% solution of N,N,N',N'-tetramethyl-p-phenylene-diamine dihydrochloride

(Sigma) dropwise onto 24 h growth on gelatin agar plates. The appearance of a blue color on the bacterial colony indicated oxidase activity.

Fermentation reactions. The capacity of each isolate to ferment arabinose, inositol, lactose, mannose, salicin, and sucrose (Sigma) was carried out in OF Basal Medium (Difco). A 10% solution of each carbohydrate was filter-sterilized and added to OF medium to give a 1% final concentration. Each carbohydrate medium was delivered into wells of tissue culture plates, overlayed with sterile mineral oil and inoculated as described above under Decarboxylase and dihydrolase reactions. The carbohydrate fermentation cultures were incubated for 7 days. Acid production in the medium indicated a positive fermentation reaction.

Gas production from glucose. Durham tubes which contained 1% tryptone (Difco), 1% NaCl, 1% glucose (Sigma), and electrolyte supplement were inoculated with each isolate. After 48 h incubation, the inverted tubes were observed for the presence of entrapped gas.

Growth at 42 ^{o}C. The ability of each isolate to grow at 42 ^{o}C was determined by streaking each isolate to a J-Agar slant. Visual growth was scored as positive following 48 h incubation at 42 ^{o}C.

Growth on sole carbon sources. Each isolate tested was propagated in the basal medium broth of Baumann et al. (1971) for 24 h before testing. Following the 24 h "starvation period," each isolate was inoculated onto the

basal medium agar formulation of Baumann et al. (1971) which was supplemented with a 0.2% concentration of the carbon source tested. The sole carbon sources used were gamma-aminobutyrate, cellobiose, citrulline, D-gluconate, D-glucuronate, L-leucine, putrescine, sucrose, D-xylose (Sigma) and ethanol (Aaper Alcohol and Chemical Company, Shelbyville, Kentucky). This assay was carried out in 24-well tissue culture plates. Each well was inoculated by placement of a drop of the basal broth suspension onto the agar surface with a sterile Pasteur pipet. The inoculated substrates were incubated for 14 days and the appearance of discernible growth was scored as positive.

Nitrate reduction. The capacity of each isolate to reduce nitrate to nitrite was determined by inoculation of each onto Nitrate Broth (Difco) supplemented with 1.5% agar. This assay was carried out in tissue culture cell well plates. After 4 days incubation, the test reagents of Edwards and Ewing (1972) were used to detect the reduction of nitrate to nitrite. These test reagents consisted of two solutions. Solution A was prepared by dissolving 8 g of sulfanilic acid in 1000 ml of 5 N acetic acid. Solution B was prepared by dissolving 5 g of alpha naphthylamine in 1000 ml of 5 N acetic acid. Immediately before use, equal parts of the solutions A and B were mixed and 0.1 ml of the mixture was added to each culture. Development of a red color at this point was scored as a positive test. The cultures which showed no color change were sprinkled with zinc dust (E. H. Sargent and Company, Chicago, Illinois) to

determine whether or not nitrite had been reduced to elemental nitrogen before addition of the test reagents. No change in color after addition of the zinc indicated a positive nitrate reduction test.

0/129 sensitivity. Sensitivity to the vibriostatic agent 0/129 (2,4-diamino-6,7-diisopropyl pteridine) was done by placing disks containing 10 and 150 ug of 0/129 onto the surface of a gelatin agar plate streaked by the Kirby-Bauer method with the isolate under test. Zones of growth inhibition indicated sensitivity. The 0/129 disks were prepared by dissolving 104.9 mg 2,4-diamino-6,7-diisopropyl pteridine phosphate (Sigma) in 10 ml sterile distilled water. The 150 ug disks were prepared by impregnation of 6.35 mm sterile paper disks (Difco) with 20 ul of the stock solution. A 20 ul micropipetter was used (Cole-Parmer Instrument Company, Chicago, Illinois). The 10 ug disks were prepared by diluting 0.67 ml of stock 0/129 in 9.33 ml sterile distilled water and sterile disks were impregnated with 20 ul of this solution. The 0/129 disks were dried at room temperature in a desiccator and stored at 4 ^{o}C under desiccation.

ONPG hydrolysis. The ability of the isolates to hydrolyze o-nitro-phenyl-beta-D-galactopyranoside with beta-D-galactosidase was determined by preparation of a saline suspension of each isolate from a 24 h KIA slant. An ONPG disk (Difco) was incubated in 0.2 ml of each suspension. Following overnight incubation in a 37 ^{o}C water bath, a color change from colorless to yellow was scored as

positive.

Salt tolerance. The ability to grow in various concentrations of NaCl was determined by observing growth in 1% tryptone broth tubes which contained 0%, 3%, 6%, 8%, and 10% NaCl. Each broth was prepared in a volumetric flask and autoclaved in sealed tubes. Visible growth in the tubes incubated for 48 h was scored as positive.

Indole production. Indole production was determined by addition of Kovac's Reagent, prepared from the formula of Edwards and Ewing (1972), into the 48 h 3% NaCl broth culture. The formation of a bright, fushia red ring at the broth surface was scored as positive.

Luminescence. The ability of each vibrio isolate to emit light was determined by the method of West and Colwell (1984). Luminescence agar plates were streaked from the 3% NaCl salt tolerance culture. Each plate was examined for luminescence in a dark room after 12, 18, and 24 hours incubation at room temperature.

Swarming. Evidence for swarming was determined by examination of bacterial growth in each quadrant of the enzyme assay plates. If the bacterial growth covered the quadrant within 48 h, the isolate was scored as a swarmer.

Vogues-Proskauer reactions. The production of acetyl-methylcarbinol was determined by the method of Furniss et al. (1978). The VP assay was carried out in 24-well tissue culture plates. The development of a "deep" red ring on the agar surface indicated a positive test.

Numerical taxonomy. The 46 differential

bacteriological characters obtained from the unknown isolates and Vibrio reference strains were subjected to numerical taxonomic analysis. Positive and negative results were encoded as 1 and 0 respectively and missing data was encoded as 9. These data were analyzed for strain similarities with the Jaccard coefficient and strains were sorted using unweighted-average-linkage clustering. The computations were carried out with the program, TAXAN Version 3.0, developed by Rita Colwell, Department of Microbiology, University of Maryland at College Park.

Relationships among all of the wild strains and the reference strains were visualized by a dendrogram. A phenogram with triangle similarities of $\geq$75% was used to group phenotypically identical and very similar strains. The phena were identified by the reference strains which were included in them.

DNA analyses. Two 100 ml starter cultures of each bacterial strain were grown in J-Broth. The J-Broth formulation was (g/l): nutrient broth, 25; NaCl, 22.5; KCl, 1; $MgCl_2 \cdot 6H_2O$, 4. The starter cultures were incubated in an incubator-shaker (Lab-Line Instruments, Orbit Floor Model) for 6 h at 30 ^{o}C and rotated at 270 rpm. A Gram stain of each starter culture was made at this point as a means of determining culture purity. The starter cultures were used to inoculate two 1-liter J-Broth cultures of each bacterial strain. The entire content of each 100 ml starter culture was used to inoculate each 1-liter culture. The 1-liter broth cultures were incubated in the

incubator-shaker for 16 h at 30 °C and rotated at 160 rpm. Before harvesting the cells, a Gram stain of each culture was made as an indicator of culture purity.

Cells were harvested by centrifugation (Sorvall centrifuge, Model RC-5B) at 13,000 x g for 20 minutes at 4 °C. The supernatant fluid was decanted and the cells were stored at -20 °C until DNA extraction was carried out.

DNA extraction. DNA was extracted and purified by the chloroform-isoamyl alcohol method of Marmur (1961). Purification of each preparation was monitored spectrophotometically. A value of 1.8 for the ratio of optical density at 260 nm to optical density at 280 nm was used to ascertain a lack of protein and ribonucleic acid contamination.

DNA base composition. The midpoint denaturation temperature (Tm) of each DNA preparation was determined in a computerized Gilford Response II UV -VIS spectrophotometer with a thermocuvette, heating block, circulating cooling water assembly, and a Microline 92 printer (Gilford Systems, Ciba Corning Diagnostics Corp. Oberlin, Ohio.)

DNA stock solutions were diluted in 0.1X standard saline citrate buffer (SSC/10) to an optical density of 0.9 at 260 nm (approximately 50 ug/ml). The SSC/10 formulation used was (g/l): NaCl, 0.87; $Na_3C_6H_5O_7 \cdot 2H_2O$, 0.44; pH 7.0 $\pm$0.2. The diluted DNA solutions were then loaded into removable quartz micro-cuvettes and the instrument was programmed for temperature increases of 1 °C/min. Hyperchromicity was monitored by the instrument and graphic

and tabular printouts of the thermal denaturation profiles were produced.

The guanine-plus-cytosine DNA base composition (mol% G + C) of each sample was calculated using the formula of Mandel et al. (1970) where

$$\text{mol\% G + C} = 100\ (\frac{\text{Tm}}{50.2} - 0.990).$$

Escherichia coli ATCC 11303 DNA was included as a control reference standard in all melting curve runs.

DNA-DNA reassociation. DNA - DNA reassociations were carried out in free solution by the method of Norgard and Bartell (1978) and as further modified by this author. The DNA stock solutions were diluted in SSC/10 to an optical density of 3.0 at 260 nm (approximately 150 ug/ml). Each sample was then sheared by being passed 10 times through a 26 gauge needle fitted to a 5 ml plastic disposable syringe, using maximum hand pressure. For each reassociation run, six tubes of DNA controls and solutions were set up as follows:

1. 0.5 ml SSC/10 (blank)
2. 0.5 ml Reference strain DNA
3. 0.5 ml Unknown DNA #1
4. 0.5 ml Unknown DNA #2
5. 0.25 ml Reference strain DNA + 0.25 ml Unknown DNA # 1
6. 0.25 ml Reference strain DNA + 0.25 ml Unknown DNA # 2

Next, 0.5 ml of a 50% (vol/vol) solution of formamide

(Eastman Kodak Co., Rochester, N.Y.) in 12X SSC buffer was added to each tube. The 12X SSC buffer formulation was (g/l): NaCl, 104.4; $Na_3C_6H_5O_7 \cdot 2H_2O$, 52.9; pH 7.0. Each preparation was sufficient for approximately 3 runs. The solutions were loaded into the micro-cuvettes of the spectrophotometer and the micro-cuvettes were then placed in the thermocuvette block assembly. The instrument was programmed to heat the DNA at a rate of 1 ^{o}C/min. up to 95 ^{o}C and to hold temperature at 95 ^{o}C for 1 minute. At this point, denaturation was complete as evidenced by the denaturation profile on the video display of the instrument. The solutions were then rapidly cooled to the preselected reassocation temperature. Cooling time was approximately 1.75 minutes. An optimal reassociation temperature of 25 ^{o}C less than the Tm of the reference strain DNA was used. In some instances, reassociations were also carried out at the stringent reassociation temperature of 15 ^{o}C less than the Tm of the reference strain DNA. DNA solutions were held at the reassociation temperature for 10 minutes. As DNA binding occurred during the reassociation time, the Gilford Response II monitored the degree of hypochromicity relative to time and graphically determined the linear rate of each reaction by plotting absorbance at 270 nm against time. The linear rate was calculated by using the linear least squares calculation $A_t = mx_t + b$ where

A = absorbance at time, t

m = rate

x_t = time increment

b = absorbance intercept

The slopes which resulted from this calculation represented an average rate (V) which is the minimized difference between the actual data points and the calculated slope (Burrer, 1985). The DNA % relative reassociation was calculated from the equation of DeLey et al. (1970) where

$$\% \text{ reassociation} = 100 \cdot \frac{4V_{mix} - (V_{DNA\ ref.} + V_{DNA\ unk.})}{2\sqrt{V_{DNA\ ref.} \cdot V_{DNA\ unk.}}}$$

Electron microscopy. Representative strains were grown for 24 h in J-Broth and on J-Agar slants. Portions of the cultures were negatively stained with a 2% (wt/vol) solution of uranyl acetate and examined on a JEOL 100CX transmission electron microscope.

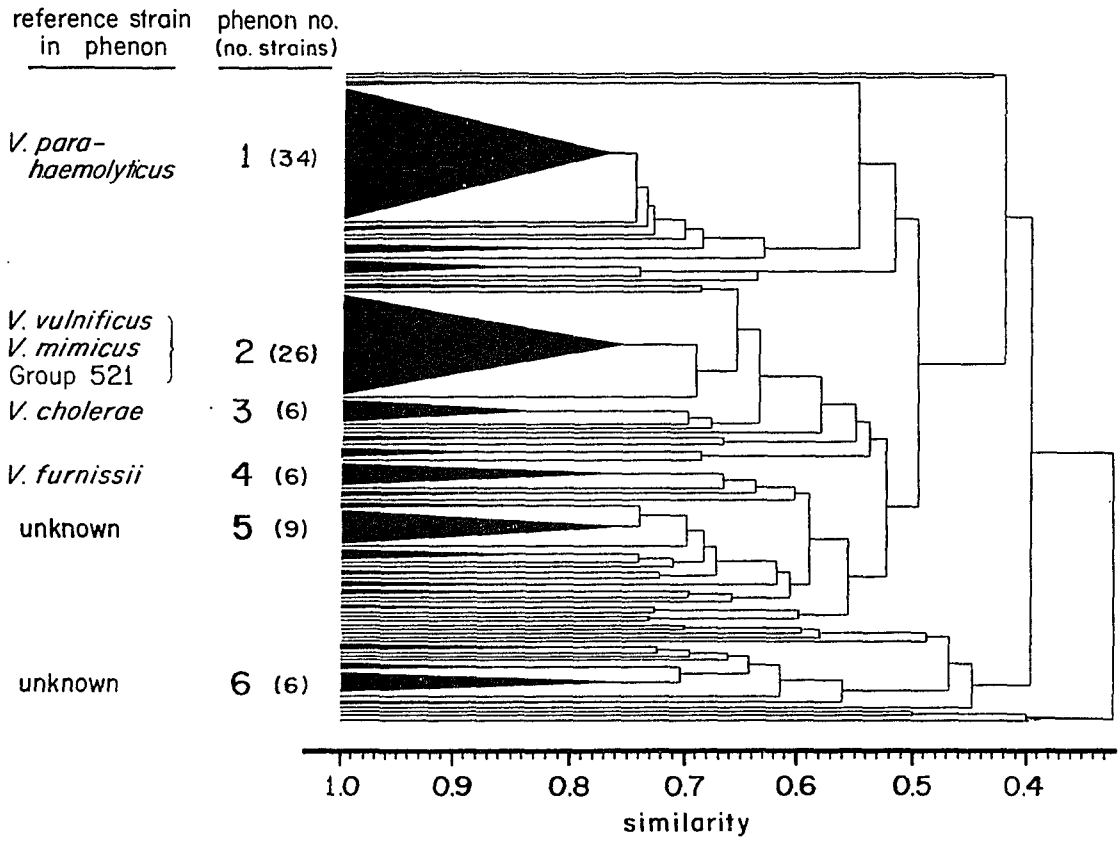
RESULTS AND DISCUSSION

Of 127 Vibrio wild strains recovered from Louisiana oysters, 101 clustered into 20 major and minor phena at ≥75% similarity when analyzed by the numerical taxonomy program TAXAN Version 3.0 (Fig. 3). A major phenon, as described in this study, is one containing six or more isolates whereas a minor phenon contains less than six isolates. Vibrio reference strains clustering into major phena with the wild isolates were V. parahaemolyticus, V. vulnificus biogroup 1, V. mimicus, Vibro Group 521, V. furnissii, and V. cholerae. Reference strains clustering into minor phena were V. alginolyticus, V. tubiashii A, V. tubiashii O, V. vulnificus biogroup 2, and Vibrio Group 510, which has been identified an as arginine-negative strain of V. diazotrophicus (J. J. Farmer, CDC, personal communication).

Human pathogens identified among the wild strains in order of prevalence were V. parahaemolyticus, V. vulnificus biogroup 1, V. mimicus, V. furnissii, V. cholerae, and V. alginolyticus. It was not surprising that these human pathogens were isolated from the oysters in this study because Roberts et al. (1982) had previously reported that V. cholerae, V. parahaemolyticus, V. fluvialis, and V. vulnificus are ubiquitous in the Louisiana coastal environment. At the time of their report, V. furnissii was considered to be an aerogenic biogroup of V. fluvialis, and later found by genetic examination to represent a new

Figure 3. Phenogram showing relationships among 127 Vibrio wild strains and 46 Vibrio reference strains based on the Jaccard coefficient and unweighted average linkage clustering. Phena are defined at 75% similarity and the 6 major phena are numbered.

Vibrio strains of Louisiana Oysters Grouped at ⩾ 75% Similarity



species (Brenner et al., 1983).

Findings seemed consistent with data reported by Farmer et al. of the CDC (1985). The six human pathogenic Vibrio species recovered from Louisiana oysters were the same species comprising 89% of 753 Vibrio isolates recovered from human sources and 91% of 291 Vibrio isolates from nonhuman or environmental sources in their study. Of 67 oyster isolates in the CDC report, V. cholerae, V. parahaemolyticus, and V. mimicus were most prevalent.

Shellfish and fish pathogens identified among the wild strains were V. tubiashii A, V. tubiashii O, and V. vulnificus biogroup 2. The fact that V. tubiashii was detected in Louisiana commercial oysters is a significant and disturbing finding, though not surprising. This organism is an etiologic agent of bacillary necrosis disease in bivalve mollusks (Tubiash et al., 1965, 1970; Hada et al., 1984). Molluscans known to be susceptible to V. tubiashii are the hard clam (Mercenaria mercenaria), the American oyster (Crassostrea virginica), the Pacific oyster (Crassostrea gigas), the European oyster (Ostrea edulis), the bay scallop (Acquipecten irradians), and the shipworm (Teredo navalis) (Tubiash et al., 1965; Hada et al., 1984). Vibrio tubiashii attacks its bivalve hosts while they are in the larval and juvenile stage. In bivalve hatcheries, the disease outbreaks are characterized by sudden onsets and heavy mortality rates (Tubiash et al., 1965). V. tubiashii has been isolated from overtly normal oysters (as was done in this study) and from the environment of healthy, adult

oysters (Tubiash et al., 1970); therefore, it has been established that this pathogen, of economic importance to the seafood industry, exists in commercial oyster beds in natural settings. It is feasible, therefore, to assume that the warm temperatures of the Louisiana summer months may provide conditions conducive to oyster spawning and bacterial proliferation concurrently. This could place quantitative limits on oyster harvests.

Vibrio vulnificus biogroup 2, an eel pathogen, was first cultured from lesions on eels (Anguilla japonica) which are grown commercially in Japan (Tison et al., 1982). The presence of this organism in Louisiana oysters has not been previously reported, but the isolation of this organism from the oysters in this study is not a surprising finding when one considers the prevalence of V. vulnificus biogroup 1 in Louisiana oysters. Vibrio vulnificus biogroup 1 is not pathogenic for eels (Tison et al., 1982), but whether or not V. vulnificus biogroup 2 is pathogenic for humans has not been established. When V. vulnificus is isolated from a patient specimen in the clinical laboratory, it is usually identified by bacteriological methods. The two V. vulnificus biogroups are distinguishable serologically but very similar phenotypically except for indole production, ornithine decarboxylase activity, growth at 42 ^{o}C, and mannitol and sorbitol fermentation reactions. Genotypically, these two biogroups exhibit >90% DNA - DNA reassociation at the stringent temperature, Tm - 15 ^{o}C (Tison et al., 1982). The possibility exists that some of

the V. vulnifcus strains isolated from human sources in a clinical laboratory could be V. vulnificus biogroup 2 and the presence of this organism in commercial oysters should not be taken lightly.

The phenogram (Fig. 3) revealed two major phena, Phenon 5 with nine isolates and Phenon 6 with six isolates, into which no Vibrio reference strains clustered. The six isolates of Phenon 6 most closely resembled Vibrio damsela bacteriologically (Fig. 4). This cluster showed a phenotypic similarity of 64.5% to the V. damela ATCC 35083 reference strain. Of the 46 bacteriological characters used to sort the wild isolates into identifiable groups, the isolates of Phenon 6 differed from the V. damsela reference strain in only three characters. The wild isolates were positive for gelatinase production and lipase production and negative in the Voges-Proskauer reaction.

DNA was extracted from each of the Phenon 6 isolates and the Tm of each preparation was determined from thermal denaturation profiles (Table 2). The DNA base composition of each isolate was computed and is shown in Table 2. The Tms of the wild strains in Phenon 6 ranged from 69.9 to 70.7 with an average Tm of 70.4. The mol% G + C contents of the wild strains ranged from 40.2 to 41.8 with an average of 41.1. These parameters were nearly identical to those of the V. damsela reference strain which had a Tm of 70.4 and a mol% G + C content of 41.2. When DNA-DNA reassociation assays were run between the DNA of the wild strains and the DNA of the V. damsela reference strain, the % relative

Figure 4. A portion of a phenogram showing the relationship between Phenon 5 and Phenon 6 and the most closely related Vibrio reference strains.

Undescribed *Vibrio* Groups and Most Closely Related *Vibrio species*

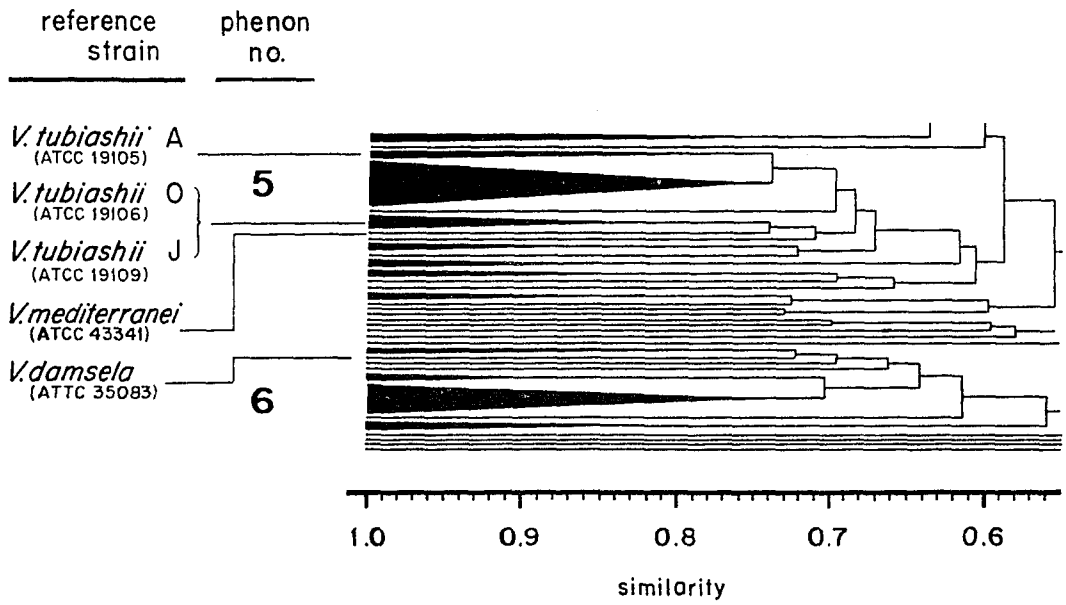


Table 2. Thermal denaturation midpoint temperatures and DNA base compositions of Phenon 6 isolates and Vibrio damsela ATCC 35083.

Strain No	Tm	mol% G + C
L15	70.3	41.0
L119	70.7	41.8
L123	69.9	40.2
L134	70.4	41.2
L141	70.4	41.2
L160	70.5	41.4
Vibrio damsela ATCC 35083	70.4	41.2

reassociations ranged from a low of 69% to a high of 96% (Table 3). Three of the strains, L15, L119, and L123, showed very high degrees of genetic homology of 94%, 96%, and 94% respectively. The strains L134, L141, and L160 showed relatively high degrees of homology of 76%, 77% and 69% respectively.

There is no universally accepted DNA percent homology above which two organisms are considered to be members of the same species. Based on data from his investigations, Johnson (1984) believes that 60 - 70% homology is a point which indicates changes in base sequences and not genetic events which are merely cistron-rearranging in nature. He therefore suggests 60% homology as a cut-off point for delineating between species. When 60% is defined as the cut-off point between species, each isolate in Phenon 6 is V. damsela.

There exist no previous reports of the isolation of V. damsela from Louisiana oysters. This organism was originally isolated from skin lesions of marine fish off the coast of California (Farmer et al., 1985) and is known to be pathogenic to damselfish, brown sharks, spiny dogfish and humans (Fernandez et al., 1975; Love et al., 1981; Grimes et al., 1984; Kreger et al., 1984; Janda et al., 1988). The reported manifestations of V. damsela infections in humans are exclusively through wound infections (Fernandez et al., 1975; Love et al, 1981; Morris et al., 1982; Kreger et al., 1984). Morris et al. (1982) reported the isolation of V. damsela from wound infections of six persons none of whom

Table 3. Reassociation of DNA from Phenon 6 oyster isolates with DNA from Vibrio damsela ATCC 35083 at optimal temperature (Tm -25 °C).

Strain No.	% relative reassociation with V. damsela DNA
L15	94
L119	96
L123	94
L134	76
L141	77
L160	69
V. damsela ATCC 35083	99

had underlying debilitating diseases which is the usual prerequisite in persons with V. vulnificus infections. Five of these individuals had been exposed to brackish or salt water. V. damsela infections can be quite severe and even fatal (Janda et al., 1988). Extreme caution should be followed in handling raw seafood and especially when performing manipulations in which wounds are easily sustained, such as while shucking oysters. Clarridge et al. (1985) reported a fatal V. damsela infection resulting from a minor laceration sustained by a 61-year old man while cleaning a catfish. Confirmation of the existence of this organism in the Louisiana coastal environment may be significant.

The nine isolates of Phenon 5 most closely resembled Vibrio tubiashii and Vibrio mediterranei (Fig. 4). Phenotypically, this group showed 74% similarity to the V. tubiashii Type A ATCC 19105 reference strain and 68.5% similarity to both V. tubiashii Type O ATCC 19106 and V. tubiashii Type J ATCC 19109. The major phenotypic differences between the Phenon 5 wild strains and the V. tubiashii reference strains were the ability of the wild isolates to grow in medium which contained 8% NaCl, their ability to grow at 42 ^{o}C, and the inability of these isolates to use citrulline as a sole carbon source.

Phenon 5 isolates showed a 68.5% phenotypic similarity to the reference strain V. mediterranei ATCC 43341. The major phenotypic differences between these isolates and V. mediterranei were the ability of the wild strains to grow at

42 °C, hydrolyze gelatin and their inability to use putrescine as a sole source of carbon.

DNA was extracted from each isolate in Phenon 5 and denaturation profiles were done. The Tms of the Phenon 5 isolates ranged from 73.3 to 74.8 with an average Tm of 73.9 for this group (Table 4). The Tms of the V. tubiashii Types A, O, and J reference strains were 71.9, 71.7, and 72.1 respectively. The Tm of the V. mediterranei reference strain was 71.6. Mol% G + C values which were calculated from the Tm of each Phenon 5 isolate ranged from 47.0 to 50.0 with an average of 48.1 for the group (Table 4). The mol% G + C values for the V. tubiashii Types A, O, and J reference strains were 44.2, 43.8, and 44.6 respectively. The V. mediterranei reference strain had a mol% G + C value of 43.6. This data shows that the DNA base composition of each of the wild isolates is richer in guanine and cytosine content than that of each of the four reference strains to which they were compared.

The %DNA - DNA hybridization value for each of the nine isolates in Phenon 5 showed $\leq$50% reassociation with the three V. tubiashii strains and the V. mediterranei reference strain (Table 5). In most cases, the degree of homology was very low. For example, isolate L98 gave a reassociation value of 1% with V. tubiashii A. The nine oyster isolates of Phenon 5 are clearly not V. tubiashii or V. mediterranei.

The nine unidentified oyster isolates which surfaced in this study appear to be a group of previously undescribed vibrios. In recent years, there have been several reports

Table 4. Thermal denaturation midpoint temperatures and DNA base compositions of Phenon 5 isolates, three Vibrio tubiashii reference strains and one Vibrio mediterranei reference strain.

Strain No.	Tm	mol% G + C
L92	74.2	48.8
L98	73.6	47.6
L111	73.8	48.0
L112	73.5	47.4
L125	73.9	48.2
L130	73.9	48.2
L137	73.3	47.0
L146	74.8	50.0
L164	73.8	48.0
V. tubiashii Type A ATCC 19105	71.9	44.2
V. tubiashii Type O ATCC 19106	71.7	43.8
V. tubiashii Type J ATCC 19109	72.1	44.6
V. mediterranei ATCC 43341	71.6	43.6

Table 5. Reassociation of DNA from Phenon 5 oyster isolates with DNA from three Vibrio tubiashii and one Vibrio mediterranei reference strains at optimal temperature (Tm - 25 °C).

Strain No.	V. mediterranei	V. tubiashii A	V. tubiashii O	V. tubiashii J
L92	17	50	10	22
L98	4	1	40	27
L111	29	12	12	24
L112	37	19	30	21
L125	49	18	11	16
L130	27	10	15	16
L137	20	35	22	23
L146	21	7	13	15
L164	17	46	31	29
V. mediterranei ATCC 43341	100			
V. tubiashii A ATCC 19105	-	100	35	39
V. tubiashii O ATCC 19106	-	-	98	76
V. tubiashii J ATCC 19109	-	40	72	87

of numerical taxonomic studies of strains of the family Vibrionaceae (Bryant et al, 1986, 1986; West et al., 1986). In each of these reports, the properties of groups of organisms that did not cluster with reference strains have been described. Bryant et al. (1986) combined data from 1091 strains in the family Vibrionaceae which had been collected from throughout the world and published in five different studies. When subjected to numerical taxonomic classification, 59 phena, containing from 2 to 86 isolates each, emerged. Of these, 14 phena housed unidentified organisms. The phenotypic characteristics of the nine oyster isolates in Phenon 5 (Fig. 4 and Table 6) do not correspond to the properties of any of Bryant's 14 unnamed groups. The isolates in Phenon 5 best resemble isolates in Phenon 12 of Bryant, but are distinct from that group in that they exhibit the ability to tolerate 8% NaCl, grow at 42 ^{o}C, hydrolyze chitin, and lack the ability to dihydolyze arginine.

The phenotypic characteristics exhibited by the Phenon 5 isolates are shown on Table 6. Isolates in this group resemble each other phenotypically to a level of $\geq$75% similarity based on the 46 characteristics listed on Table 6. The level of DNA reassociation between each of the oyster isolates and DNA of each of the four phenotypically similar Vibrio reference strains showed low homologies (Table 5), and DNA reassociations among the nine Phenon 5 isolates were not done. As a result, it is possible that more than one species exists among these nine isolates.

Table 6. Phenotypic characteristics of the nine Phenon 5 isolates. Values are the percentage of positive strains. Characters which were 100% or 0% positive have been replaced by + and - signs respectively.

Test	Phenon 5 Isolates
Catalase	+
Gas from Glucose	-
Growth at 42 ^{o}C	+
Indole	+
KIA K/A at 24 h	+
Gas in KIA	-
H_2S Production	44
Luminescence	-
Nitrate reduction	+
ONPG	33
TCBS Acid production	+
Swarming	-
Voges-Proskauer	-
Carbohydrate Fermentation:	
Arabinose	11
Inositol	-
Lactose	44
Mannose	+
Salicin	89
Sucrose	+
Decarboxylase-dihydrolase:	
Moeller's Lysine	11
Arginine	-
Ornithine	11
Enzyme Production:	
Alginase	-
Amylase	+
Chitinase	+
Gelatinase	+
Lipase (Tween 80)	+

continued

Table 6--continued

Test	Phenon 5 Isolates
Oxidase	+
Halotolerance:	
0% NaCl	-
3%	+
6%	+
8%	78
10%	-
O/129 Sensitivity:	
10 ug	+
150 ug	+
Use of sole carbon sources:	
gamma-aminobutyrate	-
cellobiose	+
citrate (Simmons)	+
citrulline	22
ethanol	-
D-gluconate	+
D-glucuronate	11
L-leucine	-
putrescine	-
sucrose	89
D-xylose	-

Table 5 shows diverse differences in reassociation values for each of the nine isolates to each V. tubiashii reference strain, from which it might be concluded that more than one species is present. This conclusion, however, cannot be made because DNA - DNA hybridizations in free solution produce only semi-quantitative values for organisms of low homology that have few and imperfectly matched DNA sequences (DeLey et al., 1970).

The isolates in Phenon 5 are Gram-negative bacilli, approximately 2.7 microns in length and 1.7 microns in width. They have a single polar flagellum when grown in broth culture (Figs. 5 and 6). These organisms sometimes produce two polar flagella when grown on an agar surface (Figs. 7 and 8). Lateral flagella were not detected.

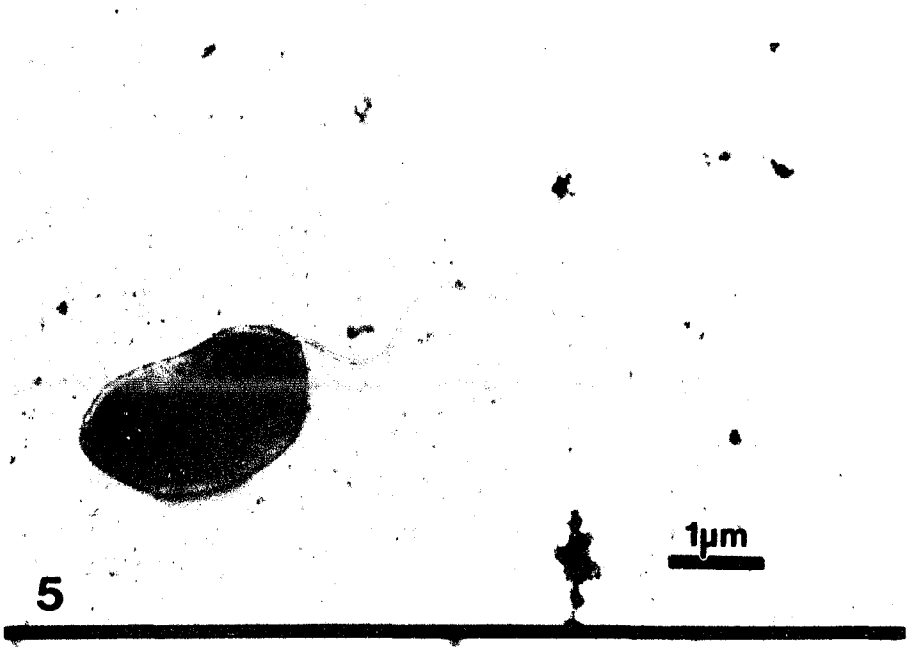
The pathogenicity for shellfish by isolates in this group of organisms remains unknown at this time. Because of their close phenotypic resemblance to Vibrio tubiashii, a known pathogen for bivalve mollusks, these organisms could prove to be of economic significance to the seafood industry.

In the numerical taxonomic grouping done in this study, V. tubiashii Type A ATCC 19105 did not cluster with V. tubiashii Type O ATCC 19106 and V. tubiashii Type J ATCC 19109. In fact, V. tubiashii Type A showed only 68.5% similarity to the V. tubiashii Types O and J reference strains (Table 5). Bryant et al. (1986) also noted that the ATCC 19105 strain failed to cluster with V. tubiashii Type O ATCC 19106 and V. tubiashii Type J ATCC 19109 strains. The

Electron micrographs of two Phenon 5 oyster isolates taken from 24 hour broth cultures.

Figure 5. Oyster isolate L137.
Magnification = 12,000.

Figure 6. Oyster isolate L111.
Magnification = 15,000.



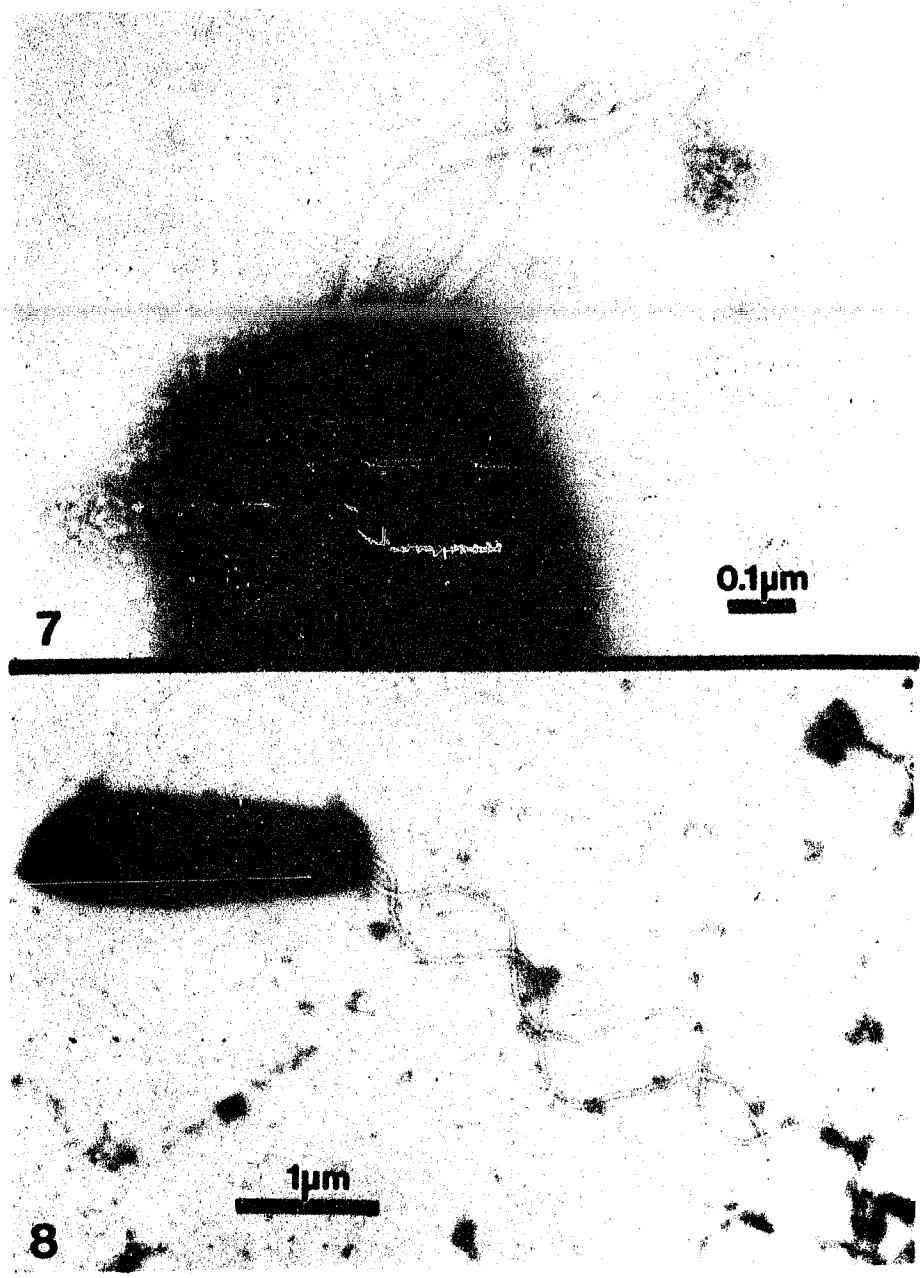
5

6

Electron micrographs of a Phenon 5 oyster isolate taken from a 24 hour culture grown on an agar surface.

Figure 7. Oyster isolate L137.
Magnification = 90,000.

Figure 8. Oyster isolates L137.
Magnification = 18,000.


0.1µm
7
1µm
8

DNA - DNA reassociation values obtained for the V. tubiashii Type A ATCC 19105 strain showed 35% relative reassociation with V. tubiashii Type O ATCC 19106 and 39% and 40% relative reassociation with V. tubiashii Type J ATCC 19109 (Table 5). Each of the three V. tubiashii reference strains used in this project were originally described by Tubiash et al. (1965) at the U. S. Bureau of Commercial Fisheries at Milford, Connecticut. The organisms were isolated from hard clam larvae, oyster spat, and juvenile hard clams and were divided into Types A, O, and J according to a serological typing scheme. Tubiash reported that strains M27 (Type O) and M74 (Type J) were morphologically and physiologically indistinquishable. Strain M17 (Type A), a highly virulent strain, differed from M27 and M74 in that it is indole negative, phenylalanine deaminase negative, produces lateral flagella when grown on an agar surface, and exhibits a somatic curvature. In 1970, each of the three strains were deposited with the American Type Culture Collection and at the time were designated as strains of Vibrio anguillarum (Tubiash et al., 1970). No molecular data was published on these strains until 1984 when Hada et al. (1984) designated these strains as a new species, Vibrio tubiashii. Hada reported 83% homology between ATCC 19105 and ATCC 19109 and 96% homology between ATCC 19106 and ATCC 19109 but did not report a level of DNA - DNA relatedness between ATCC 19105 and ATCC 19106. Though all three strains of Tubiash are serious pathogens of bivalve mollusks, the phenotypic and genotypic data of the study reported herein and the

phenotypic data of Bryant et al. (1986) indicate that these three strains could possibly comprise two separate Vibrio species.

CHAPTER II The specificity of *Vibrio vulnificus* anti-flagellar IgG and IgM monoclonal antibody coagglutination reagents.

INTRODUCTION

Vibrio vulnificus is a particularly virulent, halophilic organism which was first described in 1976 by Hollis et al. This report caused feverish excitement in the medical community which spawned a flurry of research efforts that have continued to the present. And these endeavors are well justified. Vibrio vulnificus is capable of causing death in humans within 48 to 72 hours after onset of symptoms (Hollis et al., 1976; Blake et al., 1979). This organism produces either of two disease syndromes: 1) an overwhelming primary septicemia without an obvious source of infection or 2) infection in a new or preexisting wound, which often results in secondary sepsis. Patients with V. vulnificus bacteremia frequently report recent ingestion of raw seafood, in particular oysters (Brady et al., 1984; Tacket et al., 1984; Janda et al., 1988). Patients with wound infections usually report recent exposure of the wound to seawater, brackish water, or shellfish (Blake et al., 1979, 1980; Tacket et al., 1984; Tison et al., 1986). Though otherwise healthy individuals have succumbed to V. vulnificus infections, patients with V. vulnificus infections usually have underlying liver disease, such as cirrhosis or thalassemia, which cause elevated blood iron levels (Kelly et al., 1980; Wright et al., 1981; Koga et al., 1986).

In 1982, Tison et al. conducted an extensive investigation into the identity of a vibrio isolated from

lesions of diseased eels. This organisms caused seasonal disease outbreaks in cultured eels in six different localities in Japan. Following phenotypic comparisons, eel and mouse pathogenicity studies, and DNA-DNA relatedness studies, Tison concluded that the eel pathogen was V. vulnificus which represented a different biogroup; therefore, the human pathogen was designated V. vulnificus biogroup 1 and the eel pathogen was designated V. vulnificus biogroup 2. It was determined that biogroup 1 is not pathogenic to eels, however the pathogenicity of biogroup 2 for humans is still undetermined.

Because of the extreme virulence, rapid disease course, and high mortality rate that V. vulnificus biogroup 1 produces in humans, a rapid diagnostic tool was needed. Bacteriological identification, in the classical manner, of this organism can require days and confirmation may require weeks.

It has been reported that species of the genus Vibrio possess flagellar (H) antigens that are unique to the species (Gardner et al., 1935; Sakazaki et al., 1968; Tereda, 1968; Bhattacharyya et al., 1974; Bhattacharyya, 1975; Shinoda et al., 1970; Tassin et al., 1983, 1984; Simonson et al., 1986). It is therefore feasible that species-specific anti-H reagents could be developed that would identify, in a rapid and specific manner, V. vulnificus in clinical and environmental samples.

The first attempt to develop a species-specific V. vulnificus anti-H reagent was made by Tassin and Siebeling

(1983). Polyclonal anti-H serum was produced in New Zealand White rabbits by repeated injections of Formalin-killed whole cell vaccine. The resultant H antiserum produced discernible agglutination reactions with 97.4% of V. vulnificus isolates tested. Though this anti-H serum proved to be species-specific, the assay, an H flocculation tube test, is time-consuming to perform and requires an 18 hour incubation time. More importantly, the specificity of the anti-H serum was dependent upon repeated absorption steps to remove anti-cell wall (O) and anti-capsular (K) antibody activity and also to remove anti-H activity to heterologous Vibrio species.

In 1986, Simonson and Siebeling developed a polyclonal V. vulnificus anti-H coagglutination reagent. The anti-H serum used in this reagent was produced in New Zealand White rabbits immunized with V. vulnificus flagellar core protein. The premise for using flagellar core protein as the immunogen was to eliminate the production of anti-O and anti-K activity. The coagglutination reagent was constructed by adfixing the V. vulnificus H antibody to Staphylococcus aureus cells through Fc receptors on the Staphylococcus cell wall. When anti-H, immobilized on the Staphylococcus cells, reacts with flagellar antigens, a discernible complex is formed that can be visualized in 30 to 120 seconds. In the absence of the Staphylococcus cell carrier, complexes of soluble antigens or small quantities of particulate antigen and antibody are not easily detected. The agglutination event can be adapted to a slide test thus

eliminating the labor intensive H flocculation tube test.

The polyclonal V. vulnificus anti-H coagglutination reagent identified V. vulnificus isolates with an accuracy of 99.3%. Though this reagent is quite specific, the H antiserum must be absorbed with heterologous Vibrio species to remove antibody activity directed against shared H determinants. The absorption process yields antiserum specific for V. vulnificus but it was thought that the absorption step could be eliminated by utilization of monoclonal antibody (MAbs). The premise is that a hybridoma will secrete antibody directed against a single H determinant or epitope. Simonson and Siebeling (1988) produced an anti-H latex reagent by coating immunoglobulin M(IgM) MAb, specific for V. vulnificus flagellar core protein, onto latex beads. Preliminary testing showed this reagent to be very specific.

The V. vulnificus polyclonal anti-H and monoclonal anti-H coagglutination reagents were sent to 17 Food and Drug Administration (FDA) and Public Health laboratories within the United States and Canada for assessment. One user laboratory, the FDA laboratory in Denver, Colorado, reported that an occasional vibrio isolate, recovered from East Coast oysters, that could not be identified bacteriologically as V. vulnificus agglutinated in the polyclonal anti-H reagent or the monoclonal IgM-latex reagent or both. These findings suggested that the anti-H reagents may not be species-specific. The purpose of the investigation reported here was to assess the specificity

problem the Colorado isolates presented and to assess the specificity of a coagglutination reagent produced from Staphylococcus aureus cells armed with an immunoglobulin G (lgG) MAb specific for V. vulnificus flagellar core protein.

MATERIALS AND METHODS

Source of isolates. Fifteen isolates, which had been recovered from Eastern seaboard oysters, were sent to us from the FDA laboratory in Denver, Colorado. FDA personnel reported that these isolates, designated 20709, 20730, 20749, 20751, 20752, 20757b, 20766, 20769, 20773, 20774, 20781, 20784, 20792, 20798, and 20799, had produced bacteriological reactions atypical of V. vulnificus yet each had agglutinated in the polyclonal H coagglutination reagent or the monoclonal H-latex coagglutination reagent or both.

Source of reference strains. Bacteriological, serological and numerical taxonomic analyses were carried out on 46 Vibrio reference strains in concert with the analyses of the oyster isolates. Reference strains of V. vulnificus biogroups 1 ATCC 27562 and 2 ATCC 33147 were also subjected to genetic analyses in concert with the wild strains. The reference strains used and the sources from which they were obtained are listed on Table 1.

Phenotypic characterization of oyster isolates and reference strains. Each oyster isolate and Vibrio reference strain was subjected to a bacteriological identification scheme of 46 parameters. Bacteriological tests included in the identification scheme were: sucrose fermentation in thiosulfate-citrate-bile salts-sucrose (TCBS) agar; sugar fermentation reactions, gas formation, and hydrogen sulfide (H_2S) production in Kliglers Iron Agar (KIA); citrate utilization; production of the enzymes lysine decarboxylase

Table 1. *Vibrio* reference strains used in the bacteriological, serological, taxonomic, and genetic analyses and the sources from which they were obtained.

Reference strain	Source
Vibrio aestuarianus	ATCC 35048[a]
Vibrio alginolytics	ATCC 33787
Vibrio anguillarum	ATCC 19264
Vibrio anguillarum	ATCC 14181
Vibrio campbellii	ATCC 25920
Vibrio carchariae	ATCC 35084
Vibrio cholerae Ogawa	ATCC 14035
Vibrio cholerae Inaba	LA 5875[b]
Vibrio cholerae serotype *albensis*	NCMB 41[c]
Vibrio cincinnatiensis	ATCC 35912
Vibrio Group 511 (*V. cincinnatiensis* xylose-negative)	CDC[d]
Vibrio costicola	NCMB 701
Vibrio damsela	ATCC 35083
Vibrio diazotrophicus	ATCC 33466
Vibrio Group 510 (*V. diazotrophicus* arginine-negative)	CDC
Vibrio fischeri	NCMB 1281
Vibrio fluvialis	ATCC 33810
Vibrio furnissii	ATCC 35016
Vibrio gazogenes	ATCC 29988
Vibrio harveyi	CMB 1280
Vibrio hollisae	ATCC 33564
Vibrio logei	ATCC 29985
Vibrio marinus	ATCC 15381
Vibrio mediterranei	ATCC 43341
Vibrio metschnikovii	P206
Vibrio mimicus	ATCC 33653
Vibrio natriegens	ATCC 14048
Vibrio nereis	ATCC 25917
Vibrio nigrapulchritudo	ATCC 27043
Vibrio ordalii	ATCC 33509
Vibrio orientalis	ATCC 33934
Vibrio parahaemolyticus	ATCC 10136

Continued--

Table 1--continued

Reference strain	Source
Vibrio pelagius I	ATCC 25916
Vibrio pelagius II	Unknown
Vibrio proteolyticus	MCMB 13126
Vibrio splendidus I	ATCC 33125
Vibrio splendidus II	ATCC 25914
Vibrio tubiashii A	ATCC 19105
Vibrio tubiashii 0	ATCC 19106
Vibrio tubiashii J	ATCC 19109
Vibrio vulnificus biogroup 1	ATCC 27562
Vibrio vulnificus biogroup 2	ATCC 33147
Vibrio Group 512	CDC
Vibrio Group 521	CDC
Vibrio Group 522	CDC
Baumann Group E3	ATCC 33523

a, ATCC, American Type Culture Collection, Rockville, Maryland

b, LA, Louisiana isolate

c, NCMB, National Collection of Marine Bacteria, Aberdeen, Scotland

d, CDC, Centers for Disease Control, Atlanta, Georgia

(LDC), arginine dihydrolase (ADH), ornithine decarboxylase (ODC), alginase, amylase, catalase, chitinase, gelatinase, lipase, and oxidase; fermentation to acid of arabinose, inositol, lactose, mannose, salicin, and sucrose; gas production from glucose; growth at 42 ^{o}C; utilization as single carbon sources gamma-aminobutyrate, cellobiose, citrulline, ethanol, D-gluconate, D-glucuronate, L-leucine, putrescine, sucrose, and D-xylose; nitrate reduction; sensitivity to 10 ug and 150 ug of 0/129 (2,4-diamino-6,7-diisopropyl pteridine); hydrolysis of o-nitrophenyl-beta-D-galactopyranoside (ONPG) (Both the liquid reagent and ONPG disks were used); salt tolerance in 1% tryptone broth supplemented with NaCl in concentrations of 0%, 3%, 6%, 8%, and 10%; indole production; bioluminescence; swarming; and the Voges-Proskauer reaction. The procedures used, references, and product sources are described in the Materials and Methods section, Chapter I of this dissertation.

Preparation of anti-flagellar V. vulnificus polyclonal coagglutination reagent. The polyclonal anti-H V. vulnificus coagglutination reagent was prepared by the method of Simonson and Siebeling (1986). Briefly, New Zealand White rabbits were immunized with flagellar core protein purified from V. vulnificus biogroup 1 ATCC 27562. The resulting anti-H serum was absorbed with Formalin-killed heterologous Vibrio cell suspensions to remove anti-H activity against H determinants shared by heterologous Vibrio species. The absorbed species-specific anti-V.

vulnificus IgG component was attached to the cell wall Fc receptors of Formalin-killed Staphylococcus aureus Cowan I ATCC 12598 cells by methods previously reported (Farmer et al., 1980; Simonson et al., 1986).

Preparation of anti-flagellar V. vulnificus monoclonal latex reagents. An IgM anti-H MAb-latex coagglutination reagent prepared by Simonson and Siebeling (1988) was assessed by the user laboratories. This reagent, prepared with IgM MAb from clone G7, and a coagglutination reagent made with MAb from an IgG secreting clone, F-D4, were produced by methods described by Simonson and Siebeling (1988). BALB/c mice were immunized with flagellar core protein prepared from V. vulnificus ATCC 27562 flagella. Mice which exhibited elevated anti-H serum titers were boosted with 500 ug of flagellin and sacrificed 4 days later for spleen cell collection.

B cell-myeloma cell fusions were accomplished by fusing spleen cells with log-phase Sp2/0-Ag 14 nonsecreting myeloma cells at a 4:1 ratio. Following fusion, 0.1 ml samples of a cell suspension containing 7.5×10^6 spleen cells per ml were layered over BALB/c thymocyte feeder layers in each well of a 96-well tissue culture plate. Following fusion, the hybridoma cultures were incubated at $37^{o}C$ in an humidified chamber containing 5-7% CO_2. For the first 5 days, the hybridomas were maintained in medium which contained 100 uM hypoxanthine, $4 \times 10^{-7}M$ aminopterin, and $1.6 \times 10^{-5}M$ thymidine. They were then sustained in maintenance medium, RPMI 1640, which contained 15% fetal

bovine serum, 2 mM glutamine, 1% nonessential amino acids, and 100 U of penicillin-streptomycin per ml. The supernatant fluid from each hybridoma was screened by enzyme-linked immunosorbent assay (ELISA) for V. vulnificus anti-H activity. The antigen used for this assay was a V. vulnificus flagellar core protein preparation containing 4 ug of protein per 100 ul of coupling buffer (Douillard et al., 1983). Hybridomas which secreted V. vulnificus anti-H monoclonal antibody were cloned three times by limiting dilution (Oi et al., 1980).

Monoclonal antibody was mass-produced by both batch tissue culture and in ascites fluid production. BALB/c mice were primed with 2, 6, 10, 14-tetramethyl pentadecane 10 and 3 days before intraperitoneal injection of 10^7 anti-H secreting hybridoma cells. Ascites fluid was collected from the peritoneal cavity one to two weeks following hybridoma cell injection. Hybridoma cell culture supernatant fluids and ascites fluids were sedimented to clarify the fluids. The immunoglobulins were precipitated by addition of an equal volume of saturated $(NH_4)_2SO_4$ (pH 7.0) to the clarified culture or ascites fluid and dialysed against phosphate-buffered saline KH_2PO_4, 9.118g; $K_2HPO_4 \cdot 3H_2O$, 15.29g; NaCl, 8.6g; 1000 ml distilled water; pH 7.3 (PBS). IgM MAb was purified by passage through an anti-u (mu-chain specific) agarose bead affinity column (Sigma Chemcial Co., St. Louis, MO) and the IgG MAb was purified by passage through a Protein-A affinity column (Sigma Chemical Co., St. Louis, MO).

The MAbs were isotyped by a double-antibody ELISA detection system (Hyclone Laboratories, Logan, Utah). The IgM MAb (clone G7) were attached to 0.8 um unmodified latex beads (Sigma). The latex beads were washed 3 times in glycine-buffered saline, 0.1 M glycine, 0.15 M NaCl, and 0.1% NaN_3, pH 8.2 (GBS). A 5% suspension (vol/vol) of washed latex beads in GBS was mixed with an equal volume IgM MAb 100-150 ug diluted in GBS, incubated for 2 h at 37°C and centrifuged at 1500 x g for 15 minutes. The latex bead - IgM pellet was suspended in one volume of 0.1% bovine serum albumin GBS solution.

To prepare Staphylococcus aureus Cowan I ATCC 12598 cells for IgG attachment, an 18 h broth culture was sedimented and washed three times in PBS. The washed cell pellet was suspended in 0.3% formalized PBS, incubated at room temperature for 3 hr, and washed three more times in PBS. The pelleted Staphylococcus cells were suspended in PBS to give a 10% suspension (vol/vol) and heated for 1 h at 80 °C. The Formalin-killed Staphylococcus cells were armed with the IgG MAb, in the ascites fluid, by mixing clarified ascites fluid with the 10% prepared Staphylococcus cell suspension in a ratio of 0.2 ml ascites fluid to 1 ml cell suspension. This mixture was incubated at 4 °C overnight, washed one time in PBS and the cell pellet was resuspended in 5 ml PBS.

Coagglutination. The Vibrio oyster isolates were grown on alkaline-peptone agar slants for 18-24 h at 30 °C. The alkaline-peptone agar formulation was (g/l): peptone,

10; NaCl, 20; KCl, 4; $MgCl_2 \cdot 6H_2O$, 4; yeast extract, 2; agar, 15; pH 7.4 to 7.8). The cells were harvested from agar slants in approximately 2 ml of 0.1 M Tris buffer (pH 7.8) which contained 0.1 mM ethylenediamine tetraacetic acid (EDTA) (Sigma), 1% Triton X-100, and 0.001% thimerosal (TET buffer). After 1 h and 24 h exposure to the TET buffer, a 100 ul drop of each cell suspension was placed on a glass slide next to a drop of the coagglutination reagent. The drops were mixed with a wooden applicator stick and the glass slide was tilted from side to side for 3 minutes. Indirect lighting was used as a background. A negative control was included with each test. A 100 ul drop of unarmed Staphylococcus cell suspension or uncoated latex bead suspension was mixed with a 100 ul drop of the Vibrio cell suspension. Vibrio cell suspensions which agglutinated in the unarmed coagglutination reagents were noted as "autoagglutinators" and the test results for these organisms were defined as invalid.

Numerical taxonomy. The results for each of 46 bacteriological characters obtained for each of the 15 oyster isolates and the 47 Vibrio reference strains were entered into a computer-assisted numerical taxonomic analysis. The assay results were regarded as binary characters of positive or negative. A dendrogram was constructed based on strain similarities estimated with the Jaccard coefficient and with unweighted average linkage cluster analysis. These computations were carried out with the TAXAN Version 3.0 program courtesy of Rita R. Colwell,

Maryland Biotechnology Institute, University of Maryland, College Park, MD.

DNA preparation. Selected oyster isolates, V. vulnificus biogroup 1 ATCC 27562 and V. vulnificus biogroup 2 ATCC 33147 were subjected to genetic analyses. Two 100 ml starter cultures of each bacterial strain examined were grown in J-Broth, nutrient broth, 25g; NaCl, 22.5g; KCl, 1g; $MgCl_2 \cdot 6H_2O$, 4g; 1000 ml distilled water. The starter cultures were incubated in an incubator-shaker (Lab-Line Instruments, Orbit Floor Model) for 6 h at 30 °C at 270 rpm. The starter cultures were used to inoculate each of two 1-liter J-Broth cultures for each vibrio strain. The 1-liter cultures were incubated in the incubator-shaker for 16 h at 30 °C at 160 rpm. Before harvesting, a Gram stain was made of each culture.

Cells were sedimented by centrifugation (Sorvall centrifuge, Model RC-5B) at 13,000 x g for 20 minutes at 4 °C The supernatant fluid was decanted and the cell pellets were stored frozen (-20 °C) until DNA extraction was done.

Purified DNA was prepared by a modification of the method of Marmur (1961). The cell pellets were thawed in a 37°C water bath and suspended in 25 ml of saline-EDTA buffer (0.5 M NaCl, 0.25 M EDTA, pH 8.0). The vibrio cells were lysed by addition of 10 ml 25% sodium dodecyl sulfate (SDS) Sigma), while stirring with a magnetic stir bar (Corning Laboratory Hot Plate/Stirrer, Model PC-351, Corning Glass Works, Corning, NY). The mixture was then placed in a 60 °C water bath for 10 minutes, and afterwards cooled to room

temperature. Following lysis, 5 M $NaClO_4$ (G. Frederick Smith Chemcial Co., Columbus, OH) was added to the mixture to effect a final concentration of 1 M $NaClO_4$.

Deproteinization was begun by shaking the mixture on a wrist-action shaker at a gentle setting with an equal volume of a chloroform (J. T. Baker Chemical Co., Phillipsburg, NJ)- isoamyl alcohol (Fisher Scientific Co., Fair Lawn, NJ) mixture (24:1 vol/vol) in a glass-stoppered cylinder for 30 minutes. The resulting emulsion was centrifuged at 13,000 x g for 10 minutes at 4 ^{o}C. The upper layer was removed and 2 volumes of cold (-20 ^{o}C) 95% ethanol (Quantum Chemical Corp., Deer Park, TX) were added to precipitate the nucleic acids. The precipitate was spooled and redissolved in 0.1X standard saline citrate, NaCl, 8.7g; $Na_3C_6H_5O_7 \cdot 2H_2O$ (Mallinckrodt, Inc., Paris, KY), 4.4g; 1000 ml distilled water; pH 7.0 $\pm$ 0.2 (SSC). The precipitate was dissolved by gentle rotation (approx. 100 rpm) in an incubator-shaker at room temperature for 15 minutes. The concentration of the SCC in the nucleic acid - 0.1X SSC solution was increased by adding 0.8 ml 10X SSC per 10 ml of solution. The solution was again shaken with an equal volume of chloroform-isoamyl alcohol for 15 minutes, centrifuged and the upper layer was collected. The deproteinization steps were repeated until no discernible protein was visible at the interface. The precipitate was dissolved in SCC/10 to one-half the volume removed following the last centrifugation step. A 0.2% concentration of ribonuclease (Sigma) in 0.15 M NaCl, pH 5.0, was heated at 80 ^{o}C for 10 minutes to inactivate

deoxyribonuclease. The ribonuclease was added to the nucleic acid solution to a final concentration of 50 ug/ml and the mixture was incubated at 37 ^{o}C for 30 minutes. The DNA was precipitated in cold ethanol, dissolved in 9 ml of SCC/10, and 1 ml of a 3.0 M $NaC_2H_3O_2$ (Sigma)-0.001 M EDTA mixture, pH 7.0, was added. To precipitate the DNA, two volumes of isopropanol (Sigma) were added slowly to the vortex while swirling the solution. The precipitate was washed sequentially with three changes of cold 95% ethanol to remove salt. The DNA was redissolved in SCC/10 and each preparation was examined spectrophotometrically. A value of 1.8, the ratio of optical density of 260 nm to optical density at 280 nm, was used to ascertain that protein and ribonucleic acid had been removed.

DNA base composition. The midpoint denaturation temperature (Tm) of DNA from representative oyster strains and the reference strains V. vulnificus biogroup 1 ATCC 27562 and V. vulnificus biogroup 2 ATCC 33147 were used to calculate the guanine-plus-cytosine content (mol% G + C). Denaturation was carried out in SSC/10 with increasing temperature increments of 1 ^{o}C/min. which was measured spectrophotometrically (Gilford Response II UV-VIS Spectrophotometer and Microline 92 Printer, Gilford Systems, Ciba Corning Diagnostics Corp., Oberlin, OH). Calculations were made using the formula of Mandel et al. (1970), where

$$\text{mol\% G + C} = 100\left(\frac{\text{Tm}}{50.2} - 0.990\right)$$

Escherichia coli ATCC 11303 DNA was included in each assay as a control reference standard.

DNA-DNA reassociation. DNA-DNA reassociations were carried out in free solution by the method of Norgard and Bartell (1978) and as further modified by this author (Materials and Methods, Chapter I, this dissertation). Briefly, DNA stock solutions were diluted in SSC/10 to approximately 150 ug/ml and sheared by 10 sequential passages through a 26 gauge needle. In each reassociation experiment, six tubes were set up as follows: 1) 0.5 ml SSC/10 (Blank) 2) 0.5 ml reference strain DNA 3) 0.5 ml unknown DNA #1 4) 0.5 ml unknown DNA #2 5) 0.25 ml reference strain DNA + 0.25 ml unknown DNA #1 6) 0.25 ml reference strain DNA + 0.25 ml unknown DNA #2. The DNA solutions were diluted by half with a 50% solution of formamide (Eastman Kodak Co., Rochester, NY) in 12X SSC buffer (vol/vol). Aliquots of the solutions were transferred to quartz micro-cuvettes and heated in the Gilford Response II spectrophotometer at a rate of 1 ^{o}C/min up to 95 ^{o}C. They were held at 95 ^{o}C for 1 minute and cooled rapidly to the optimal reassociation temperature of 48 ^{o}C which is approximately 25 ^{o}C less than the Tm of the two V. vulnificus reference strains used in this study. Each solution was held at the reassociation temperature for 10 minutes during which time the Gilford Response II monitored the degree of hypochromicity relative to time. The rate (V) of each reaction was determined graphically and the DNA percent relative reassociations were calculated from

the equation of DeLey et al. (1970) where

$$\% \text{ reassociation} = 100 \cdot \frac{4V_{mix} - (V_{DNA\ ref.} + V_{DNA\ unk.})}{2\sqrt{V_{DNA\ ref.} \cdot V_{DNA\ unk.}}}$$

Immunoelectron microscopy. Representative oyster vibrio isolates were stained by the immunogold technique of Adams (Doctoral dissertation, Department of Microbiology, Louisiana State University, 1987). This procedure was carried out by Laynette H. Spring. Cells to be examined were harvested in 0.15 M NaCl from 18 - 24 h J-Agar slants. One 25 ul drop of each cell suspension was placed on a parlodion and carbon coated copper grid and incubated for 15 minutes at room temperature. Each grid was drained of excess fluid and then 25 ul of 2% bovine serum albumin were placed on the cells immobilized on the grids for 15 minutes to block non-specific binding sites. Each grid was drained and 25 ul of the anti-H MAb solution was placed on the cells and incubated for 30 minutes at room temperature. Each grid was washed with 3 sequential additional drops of PBS, for 3 minutes each. Next, 25 ul goat anti-mouse immunoglobulin, both IgG and IgM, conjugated with 10 nm colloidal gold particles (Janssen Biotech N. V., Olen, Belgium) was added and incubated on each grid for 30 minutes at room temperature. The grids were washed three times in PBS and two times in deionized water. Each specimen was examined on

a JEOL 100CX transmission electron microscope.

RESULTS AND DICUSSION

The bacteriology of the oyster isolates. The fifteen oyster isolates which the Denver FDA laboratory found to exhibit atypical bacteriological profiles, yet to agglutinate in the anti-V. vulnificus H coagglutination reagents were forwarded to our laboratory. Upon receipt, each isolate was subjected to a bacteriological identification scheme of 46 parameters as described above. In agreement with the user laboratory, we found that the Colorado isolates did not conform to phenotypic traits generally attributed to V. vulnificus biogroups 1 or 2.

The isolates were divided into eight groups according to their aberrant phenotypic traits (Table 2). Isolates placed in Groups I, II, and III were H_2S producers, ONPG negative, failed to ferment lactose, and failed to decarboxylate lysine. These traits are antithetical to those of V. vulnificus although caution must be used in considering lactose fermentation as a definitive characteristic of V. vulnificus. Reichelt et al. (1976) found wild strains of this species to be unable to utilize lactose but, after growth on complex medium, some mutant strains readily acquire the ability to use this carbohydrate.

The single isolate in Group IV did not produce H_2S but was similar to isolates of Groups I, II, and III in its inability to utilize lactose, decarboxylate lysine, and hydrolyze ONPG. Members of Groups V and VI utilized

Table 2. Grouping of the oyster isolates according to their exhibited phenotypic traits which are atypical of V. vulnificus biogroups 1 and 2.

Group No.	No. of isolates	Trait
I	2	H_2S +, ONPG -, alginase +, amylase -, lactose -, salicin -, LDC -
II	2	H_2S +, ONPG -, amylase -, lactose -, mannose -, LDC -
III	1	H_2S +, ONPG -, amylase -, growth 42 °C -, lactose -, salicin -, mannose -, LDC -, ethanol +
IV	1	ONPG -, lactose -, salicin -, LDC -, ethanol +
V	1	LDC -, gamma-aminobuty-rate +, luminescence +
VI	2	gamma-aminobutyrate +, L-leucine +
VII	2	sucrose +, Voges-Proskauer +, salicin -, citrulline +
VIII	4	ONPG -, sucrose +, ethanol +

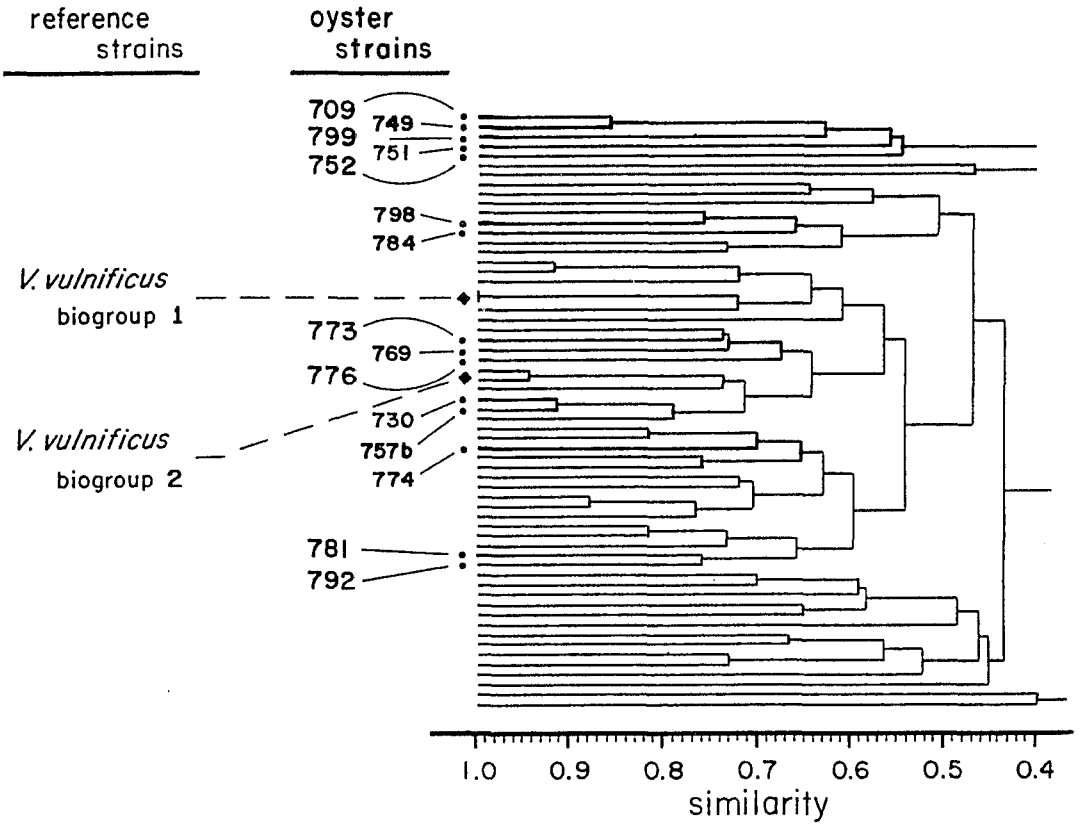
gamma-aminobutyrate as a single carbon source. The inability to utilize this substrate as a single carbon source is one of the primary characteristics which distinquishes V. vulnificus from other Vibrio species (Baumann et al., 1984). Two strains, 20730 and 20757B, were bioluminescent. While bioluminescence is not common among strains of V. vulnificus, Oliver et al. (1986) described a bioluminescent strain of V. vulnificus biogroup 1 as the causative agent of a fatal wound infection.

Members of Groups VII and VIII fermented sucrose to acid. While V. vulnificus is generally considered to be sucrose negative, Hollis et al. (1976) reported that 3% of the clinical V. vulnificus isolates which she examined were sucrose positive and Oliver reported that 15% of V. vulnificus environmental strains isolated were sucrose-positive (personal communication). The sucrose-positive isolate 20781 (Group IV) was Voges-Proskauer positive. The inability of V. vulnificus to produce acetoin is presently considered to be a definitive feature of this species.

The 46 bacteriological characteristics of the 15 oyster isolates and of 47 Vibrio reference strains which included all described species and strains reported to date were analyzed by the numerical taxonomy program TAXAN Version 3.0. The taxonomic relationship of each oyster isolate to each other and to each Vibrio reference strain is illustrated by the dendrogram in Figure 1. Three of the oyster isolates showed a phenotypic similarity of >75% to the Vibrio reference strains. Isolates 20730 and 20757b

Figure 1. A dendrogram showing the relationships of 15 oyster isolates to V. vulnificus biogroups 1 and 2 and 43 heterologous Vibrio reference strains based on the Jaccard coefficient and unweighted average linkage methods of numerical taxonomy.

Numerical Taxonomic Relationships of Oyster Isolates to *Vibrio vulnificus* biogroups and 40 *Vibrio* species



showed 79% phenotypic similarity to Vibrio Group 522, an unnamed group of lactose-positive, sucrose-negative vibrios on deposit at the CDC. Isolate 20798 showed a 76% phenotypic similarity to Vibrio parahaemolyticus. The isolates which comprised Groups I, II, and III, (strains 20709, 20749, 20751, 20752, and 20799) showed 34% similarity to any of the Vibrio reference strains. The remaining oyster isolates showed $\leq$74% similarities to the reference strains, suggesting very low bacteriological resemblance to the established Vibrio species. The percent phenotypic similarity of each oyster isolate to V. vulnificus biogroups 1 and 2 are shown on Table 3. The resemblance of these strains to the accepted bacteriological profile of V. vulnificus was low.

Two individuals in two separate laboratories located 120 miles apart subjected the V. vulnificus biogroup 1 ATCC 27562 and V. vulnificius biogroup 2 ATCC 33147 reference strains to the 46 bacteriological tests used in this investigation. The dendrogram generated by numerical taxonomic analysis revealed 100% agreement between the two sets of bacteriological data for V. vulnificus biogroup 1 and 95% agreement between the two sets of data for V. vulnificus biogroup 2 (Fig. 1 and Table 3). The percent phenotypic similarity between V. vulnificus biogroups 1 and 2 was 56% (Table 3). Tison et al. (1982) found that the major phenotypic difference between strains of V. vulnificus biogroup 1 and biogroup 2 was that V. vulnificus biogroup 2 was negative for indole production whereas

Table 3. Phenotypic similarity percentages of 15 oyster isolates to *V. vulnificus* biogroups 1 and 2.

Group No.	Strain No.	Percent similarity to *V. vulnificus* biogroup 1 ATCC 27562	Percent similarity to *V. vulnificus* biogroup 2 ATCC 33147
I	20709	34	34
	20749	34	34
II	20751	34	34
	20752	34	34
III	20799	34	34
IV	20766	57	64
V	20730	57	71
VI	20757b	57	71
	20784	47	47
VII	20773	57	64
	20781	54	54
VIII	20769	57	64
	20774	54	54
	20792	54	54
	20798	47	47
V. vulnificus biogroup 1 ATCC	27562	100	56
V. vulnificus biogroup 2 ATCC	33147	56	95

biogroup 1 isolates were positive for this trait. It was also reported that V. vulnificus biogroup 1 isolates were positive for ornithine decarboxylase activity, growth at 42 ^{o}C, and acid production from mannitol and sorbitol and the biogroup 2 strains were negative for these characters. The results of our bacteriological survey were in general agreement with those of Tison. In addition, we found that our V. vulnificus biogroup 2 reference strain was positive for chitinase activity whereas the biogroup 1 reference strain was not and that our biogroup 2 reference strain could use cellobiose, D-gluconate, and D-glucuronate as sole carbon sources whereas our biogroup 1 reference strain was unable to use these compounds. Tison did not report chitinase, cellobiose, D-gluconate, or D-glucuronate results. In Tison's investigation, DNA - DNA competition experiments revealed >90% relative reassociation between V. vulnificus biogroup 1 strains and strains of V. vulnificus biogroup 2. These findings suggest that bacteriological test results may be unreliable as diagnostic tools and should be supported by serological or genetic data.

The serology of the oyster isolates. The Denver FDA laboratory reported (personal communication) that the fifteen oyster isolates agglutinated in the polyclonal H coagglutination reagent, the IgM MAb-latex coagglutination reagent or both. When retested in our laboratory, isolates in Groups 1 and II agglutinated with the IgM MAb-latex reagent and also with uncoated control latex reagent (Table 4). Group II isolates also agglutinated in the IgG MAb - S.

Table 4. Serological reactions of 5 oyster isolates in V. vulnificus anti-H IgG (F-D4) MAb - latex, IgG (F-D4) MAb-S. aureus, and IgM (G7) MAb-latex coagglutination reagents.

Group No.	Strain No.	Agglutination reaction for anti-H MAb[a]: IgG-latex	Uncoated latex control	IgG-S. aureus	Unarmed S. aureus control	IgM-latex	Uncoated latex control
I	20709	0	0	0	0	+++	+++
	20749	0	0	0	0	+++	+++
II	20751	0	0	+	+	+++	+++
	20752	0	0	+	+	+++	+++
III	20799	0	0	0	0	0	0
IV	20766	++	0	+	0	++	0
V	20730	nd[b]		nd		++	0
VI	20757b	nd		nd		+++	0
	20784	nd		nd		+++	0
VII	20773	+++	0	++	0	+++	0
	20781	+++	0	++	0	+++	0
VIII	20769	nd		nd		++	0
	20774	nd		nd		++	0
	20792	nd		nd		++	0
	20798	0	0	0	0	0	0
V. vulnificus biogroup 1		+++	0	++	0	+++	0
V. vulnificus biogroup 2		++	0	+	0	+++	0

a, +++, coagglutination reaction within 30 seconds or less
++, coagglutination reaction within 30 to 120 seconds
+, coagglutination reaction within 2 to 3 minutes
0, no discernible coagglutination reaction within 3 minutes
b, nd, not done

aureus regent and with unarmed control S. aureus cells (Table 4). Organisms which agglutinate in control reagent, lacking antibody, are deemed "autoagglutinators" and do not yield valid serological results. Some organisms will autoagglutinate in sterile saline. It is essential that negative controls be included when serological reagents are utilized as diagnostic tools.

The isolates of Groups I and II did not agglutinate in either the IgG MAb - latex or the IgG MAb - S. aureus reagents and the Group II isolates failed to agglutinate in the IgG MAb - latex reagent. The single isolate in Group III, 20799 did not agglutinate in the IgM MAb - latex, the IgG MAb - latex, or in the IgG MAb - S. aureus reagents (Table 4) upon repeated testing by different individuals at different times. We disregarded the isolates of Groups I, II, and III as Vibrio vulnificus. Except for isolate 20798, the isolates in Groups IV, V, VI, VII, and VIII, agglutinated in the IgM MAb-latex reagent and exhibited "smooth" negative reactions (Table 4). Isolate 20798 in Group VIII did not agglutinate in any of the coagglutination reagents while the sole isolate of Group IV and both isolates of Group VII agglutinated in the IgG MAb - latex and in the IgG MAb - S. aureus reagents in addition to the IgM MAb - latex reagents. These organisms did not agglutinate in the unarmed control reagents (Table 4). The isolates in Groups IV, V, VI, VII, and VIII had exhibited limited phenotypic similarity to V. vulnificus biogroups 1 or 2.

The presence of species-specific antigen on the flagella of Vibrio species was first suggested by Gardner and Venkatraman (1935). This finding was confirmed and extended by Sakazaki et al. (1968), Terada (1968), Shinoda et al. (1970), Bhattacharyya et al. (1974), Shimada et al. (1983), Tassin et al. (1983), and Simonson et al. (1986). Polyclonal anti-H serum, raised in rabbits immunized with whole cell vaccines, must be absorbed exhaustively to remove anti-O (cell wall) and anti-K (capsular) antibody activity (Tassin et al., 1983). Even when purified flagellar core protein is used as the immunogen, polyclonal anti-H serum must be absorbed with Formalin-killed heterologous Vibrio species to remove anti-H activity against "common" H determinants (Simonson et al., 1986).

By definition, anti-H monoclonal antibody secreted by a hybridoma should express specificity to a single H determinant. Hybridomas secreting MAb to "shared" H determinants perhaps an internal antigen, could have been selected. For this reason, each anti-H reagent, the IgM (clone G7) - latex coagglutination reagent, the IgG (clone F-D4) - S. aureus coagglutination reagent, and an IgG (clone F-D4) - latex coagglutination reagent were tested against 31 heterologous Vibrio species (Table 5). One cross reaction with a heterologous species was noted. Vibrio furnissii cells were agglutinated in the IgG-S. aureus coagglutination reagent within 2 minutes. A 1:20 dilution of IgG was used to arm the S. aureus cells. The cross reaction was not seen with the IgG-latex reagent armed with a 1:200 dilution of

Table 5. Serological survey of the coagglutination of homologous and heterologous Virbrio spp. with V. vulnificus anti-H MAb reagents

Vibrio species	Agglutination reaction[a] for coagglutination reagents:		
	IgG (F-D4)-S. aureus	IgG (F-D4)-latex	Igm (57)-latex
V. aesturarianus	0	0	0
V. alginolyticus	0	0	0
V. anguillarum	0	0	0
V. campbellii	0	0	0
V. cholerae	0	0	0
V. cincinnatiensis	0	0	0
V. costicola	0	0	0
V. damsela	0	0	0
V. diazotrophicus	0	0	0
V. fisheri	0	0	0
V. fluvialis	0	0	0
V. furnissii	+	0	0
V. gazogenes	0	0	0
V. harveyi	0	0	0
V. hollisae	0	0	0
V. logei	0	0	0
V. mediterranei	0	0	0
V. metschnikovii	0	0	0
V. mimicus	0	0	0
V. natriegens	0	0	0
V. nereis	0	0	0
V. ordalii	0	0	0
V. orientalis	0	0	0
V. parahaemolyticus	0	0	0
V. pelagius	0	0	0
V. proteolyticus	0	0	0
V. splendidus I	0	0	0
V. splendidus II	0	0	0

Continued --

Table 5 - Continued

Vibrio species	Agglutination reaction for coagglutination reagents:		
	IgG (F-D4)-*S. aureus*	IgG (F-D4)-latex	Igm (57)-latex
V. tubiashii A	0	0	0
V. tubiashii O	0	0	0
V. tubiashii J	0	0	0
V. vulnificus 1	++	+++	+++
V. vulnificus 2	+	++	+++

a, +++, reaction within 30 seconds or less
++, reaction within 30 to 120 seconds
+, reaction within 2 to 3 minutes
0, no discernible reaction within 3 minutes

IgG. It is possible that V. furnissii expresses a minor H determinant similar to the "species-specific" V. vulnificus H determinant, and the cross reaction was not detectible in the higher antibody dilutions.

In an effort to determine further the specificity of the anti-H coagglutination reagents, the immunoelectron microscopy gold staining technique was used. Strains 20781 and V. vulnificus biogroup 1 ATCC 27562 (agglutinators), strains 20709, 20749, 20751, and 20752 (autoagglutinators), and strain 20798 (a nonagglutinator) were incubated with anti-H IgG (clone F-D4). Each was stained with a second antibody-colloidal gold conjugate. The gold probe consisted of goat anti-mouse IgG-IgM labeled with colloidal gold.

Through electron microscopy, it could be observed that the V. vulnificus anti-H MAb bound specifically to the flagella of the V. vulnificus biogroup 1 ATCC 27562 reference strain (Fig. 2) and to the flagella of oyster isolate 20781 (Figs. 3 and 5). Anti-H antibody did not bind to the cell wall. Anti-H MAb did not react with the autoagglutinators, oyster isolates 20709 (Fig. 8), 20749 (Figs. 6 and 10), 20751 (Fig. 9), or 20752 (Fig. 7). Nor was there any antibody affinity for oyster isolate 20798 (Fig. 4), the nonagglutinator. These data substantiate the coagglutination reactions reported on Table 4 and further reveal specificity of the anti-H reagent for the flagella of V. vulnificus.

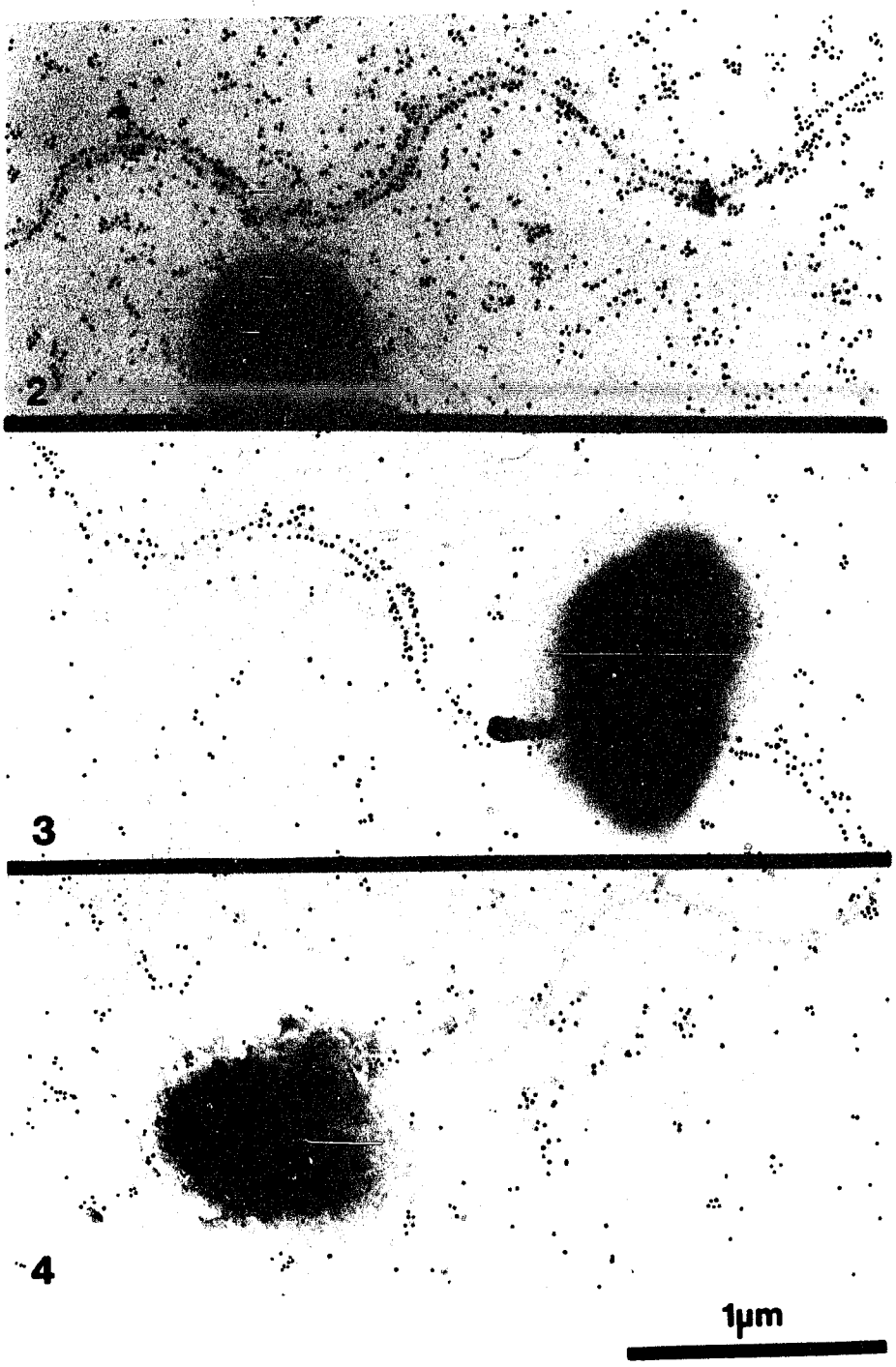
The genetics of the oyster isolates. DNA was extracted from a representative isolate in each of Groups

Immunoelectron microscopy of a Vibrio vulnificus reference strain and two oyster isolates stained with anti-V. vulnificus H MAbs and anti-mouse Ig-colloidal gold conjugate.

Figure 2. V. vulnificus biogroup 1 ATCC 27562. Magnification = 35,000.

Figure 3. Isolate 20781, an agglutinator. Magnification = 35,000.

Figure 4. Isolate 20798, a nonagglutinator. Magnification = 35,000.

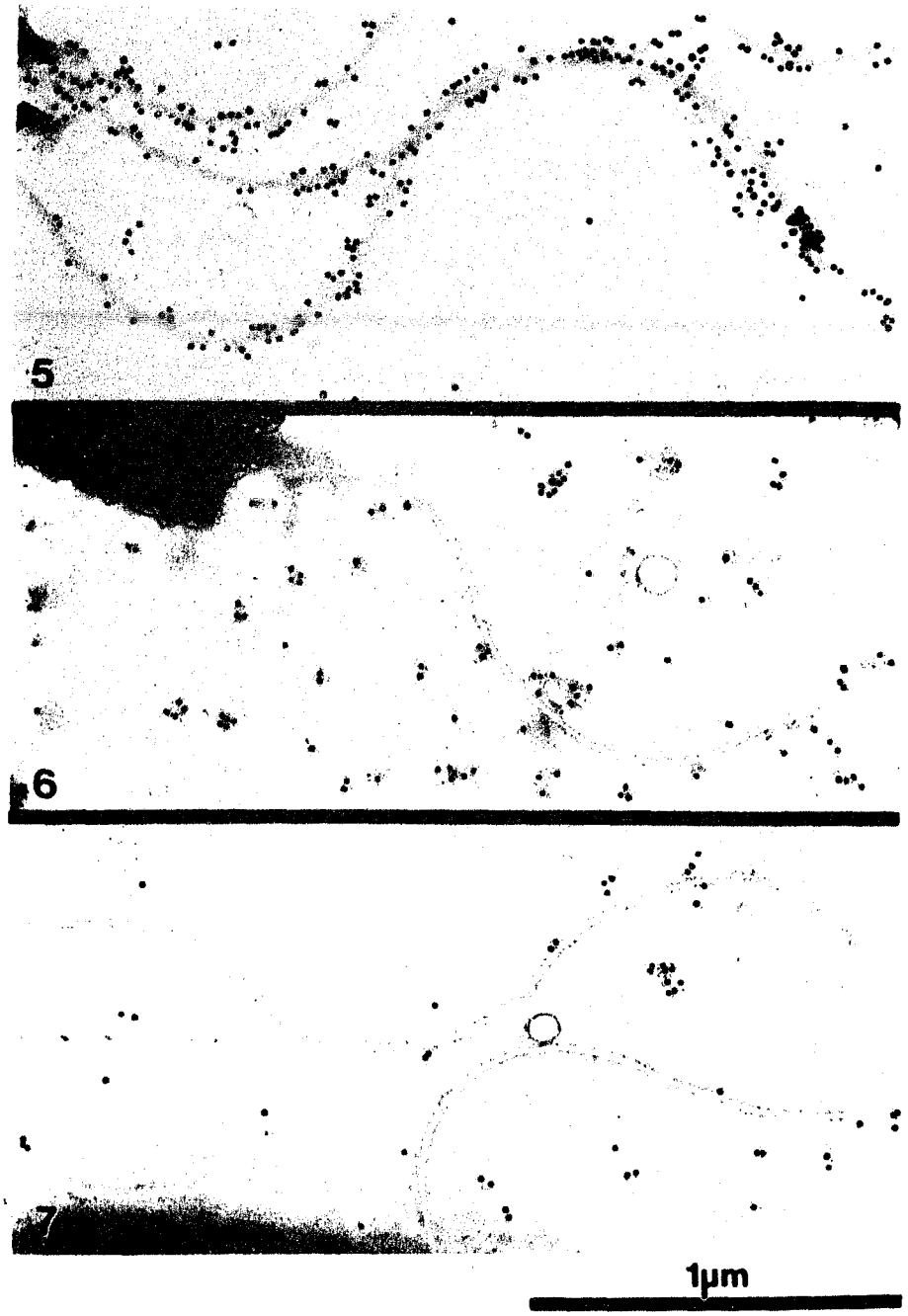

2
3
4
1µm

Immunoelectron microscopy of three oyster isolates stained with anti-*V. vulnificus* H MAbs and anti-mouse Ig-colloidal gold conjugate.

Figure 5. Flagella of Isolate 20781, an agglutinator. Magnification = 50,000.

Figure 6. Isolate 20749, an autoagglutinator. Magnification = 50,000.

Figure 7. Flagella of Isolate 20752, an autoagglutinator. Magnification = 50,000.

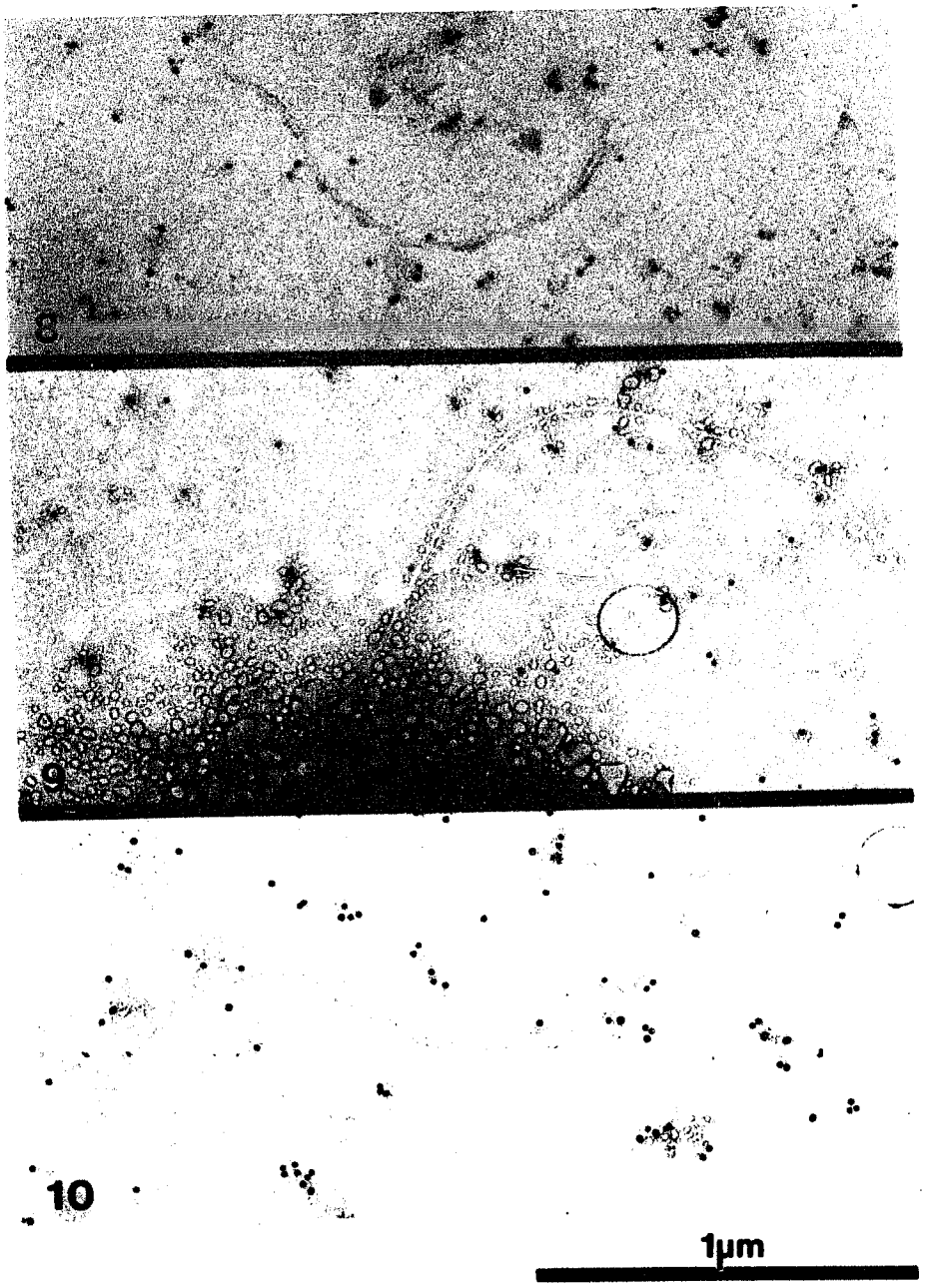

5
6
7
1µm

Immunoelectron microscopy of three oyster isolates stained with anti-V. vulnificus H MAbs and anti-mouse Ig-colloidal gold conjugate.

Figure 8. Flagellum of Isolate 20709, an autoagglutinator. Magnification = 50,000.

Figure 9. Flagellum of Isolate 20751, an autoagglutinator. Magnification = 50,000.

Figure 10. Flagellum of Isolate 20749, an autoagglutinator. Magnification = 50,000.


8
9
10
1µm

IV, V, VI, VII, and VIII and from V. vulnificus biogroup 1 ATCC 27562 and V. vulnificus biogroup 2 ATCC 33147. The Tm, mol% G + C contents (Table 6) and DNA-DNA reassociation value between the DNA of the oyster isolates and the DNA of each of the reference V. vulnificus biogroups was determined (Table 7). High DNA - DNA reassociation values were obtained for each isolate tested from Groups IV, V, VI, and VII. Based on the value of 60% genetic relatedness suggested by Johnson (1984), for delineating between species, the isolates in those groups are clearly V. vulnificus.

Isolate 20798 showed a low degree of genetic relatedness to the V. vulnificus reference strains and has been identified as V. parahaemolyticus. This isolate did not agglutinate in the V. vulnificus MAb coagglutination reagents and showed a 76% phenotypic similarity to the V. parahaemolyticus reference strain in the taxonomic analysis. It was also noted that isolate 20798 exhibited swarming on agar surfaces which is a characteristic associated with the presence of lateral flagella. Simonson and Siebeling (1986) noted that an occasional V. parahaemolyticus isolate, about 1 in 200, coagglutinated with V. vulnificus polyclonal anti-H serum.

The level of DNA-DNA association exhibited between the V. vulnificus reference strains and the oyster isolates, which agglutinated in the anti-H MAb coagglutination reagents yet showed a phenotypic dissimilarity to V. vulnificus, confirmed the species-specificity of the MAb

Table 6. Thermal denaturation midpoint temperatures and DNA base compositions of five representative oyster isolates and V. vulnificus biogroup 1 ATCC 27562 and V. vulnificus biogroup 2 ATCC 33147.

Group No.	Strain No.	Tm	mol% G + C
IV	20766	73.0	46.4
V	20730	72.6	45.6
VI	20757b	72.8	46.0
VII	20781	72.8	46.0
VIII	20798	72.2	44.8
	V. vulnificus biogroup 1	72.2	44.8
	V. vulnificus biogroup 2	72.1	44.6

Table 7. Reassociation of DNA from representative oyster isolates with DNA from V. vulnificus biogroups 1 and 2.

Group No.	Strain No.	Relative % reassociation with:	
		V. vulnificus biogroup 1 ATCC 27562	V. vulnificus biogroup 2 ATCC 33147
IV	20766	79	100
V	20730	87	79
VI	20757b	86	84
VII	20781	95	100
VIII	20798	49	31
	V. vulnificus 1 ATCC 27562	100	
	V. vulnificus 2 ATCC 33147		100

coagglutination reagents. It is believed that the IgM MAb (clone G7) and the IgG MAb (clone F-D4) possess exquisite specificity for V. vulnificus flagellar determinants and that the coagglutination reagents prepared with these MAbs may be employed as a reliable diagnostic tool for identifying strains of V. vulnificus, even those strains with atypical bacteriological profiles.

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VITA

Mary Lynelle Ford was born November 28, 1943, in Alexandria, Louisiana to Mabel and Morgan Ford. She attended public school in Pineville, Louisiana and graduated from Pineville High School in May, 1961. She entered college in September, 1961, and graduated in January, 1965, from Northwestern State College of Louisiana with a B.S. degree in Co-Departmental Science Education. From January, 1965, through July, 1966, she served as a classroom teacher of biology and chemistry at Bolton High School, Alexandria, Louisiana. In September, 1966, she entered the Graduate School of Northwestern State College and in August, 1968, was awarded a Master of Science degree in zoology.

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Mary Lynelle Ford

Major Field: Microbiology

Title of Dissertation: The Incidence of Vibrio Species in Louisiana and Maryland Oysters (Crassostrea virginica)

Approved:

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EXAMINING COMMITTEE:

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